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[Continued on next page]

(54) Title: HYPOXIA-RELATED HUMAN GENES, PROTEINS, AND USES THEREOF

A

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CGGAAGCCGG TTGGGGTGTG AGAGGTTTTC TCGCTCTAGG GAGATTCTTC
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GAAGATCAGG GATCAAAACT CATTCGAAAA GCTAAAGAGG CACCATTCGT
ACCCGTTGGA ATAGCGGGTT TTGCAGCAAT TGTTGCATAT GGATTATATA
AACTGAAGAG CAGGGGAAAT ACTAAAATGT CCATTCATCT GATCCACATG
CGTGTGGCAG CCCAAGGCTT TGTTGTAGGA GCAATGACTG TTGGTATGGG
CTATTCCATG TATCGGGAAT TCTGGGCAAA ACCTAAGCCT TAGAAGAAGA
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TATTAAAGTT ACCTATTATT GTTGGAAATA AACTAATTTG TATGGGTTTA
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GAATTTGCAT TCCATGGTGT TAACATGGTA TATGTATTGT TATTAAAGTA
AGTGACCCAT GTC
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B

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RGNTKMSIHL IHMRVAAQGF VVGAMTVGMG YSMYREFWAK PKP
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(57) Abstract: The polynucleotide and polypeptide sequences of two novel hypoxia-inducible human genes, *HIG1* and *HIG2*, are described. In addition, a number of known genes and ESTs have now been established as being hypoxia-inducible and hypoxia-repressible. Polynucleotide and polypeptide arrays comprising the hypoxia-inducible and hypoxia-repressible gene sequences, proteins, or antibodies which specifically bind the proteins are disclosed. Methods for using the hypoxia-inducible and hypoxia-repressible gene sequences and proteins, and arrays thereof, to diagnose and treat hypoxia-related conditions such as cancer and ischemia are also provided.

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HYPOXIA-RELATED HUMAN GENES, PROTEINS, AND USES THEREOF

BACKGROUND OF THE INVENTION

a) Field of the Invention

The present invention relates to hypoxia-inducible and hypoxia-repressible genes, and fragments thereof, and to the use of these sequences in the diagnosis and treatment of disease conditions involving hypoxia, including stroke, heart attack, and cancer.

b) Description of Related Art

Hypoxia is responsible for regulating a number of cellular and systemic processes, including angiogenesis, erythropoiesis, and glycolysis. Hypoxic insult and hypoxia-induced gene expression also play a role in a variety of severe pathological conditions including ischemia, retinopathy, neonatal distress, and cancer.

Hypoxia-induced gene expression is associated with ischemia (and reperfusion) in many tissues including the liver, heart, eyes, brain, and vasculature. Many of the hypoxia-induced genes are believed to be involved in the protection or repair of the cells exposed to hypoxia. Enhancement of the body's protective expression of some stress-induced genes is therefore likely to be beneficial in many ischemia/reperfusion-related conditions such as liver transplantation, bypass operations, cardiac arrest, and stroke. For instance, in the brain, the response to brain ischemia includes the enhanced expression of growth factors and anti-apoptosis genes (Koistinaho et al. (1997) *Neuroreport* 20:i-viii).

However, the ischemic induction of gene expression is not always favorable. For example, brain ischemia can also result in the expression of apoptosis genes or other genes which promote degeneration of the neuronal cells. Ischemia can also induce an extreme inflammatory reaction in the injured brain via the

upregulation of proinflammatory cytokines, chemokines, and endothelial-leukocyte adhesion molecules (Feuerstein et al. (1997) *Ann. N.Y. Acad.Sci.* 15:179-93). There is some evidence that this hypoxia-induced inflammatory response is a major cause of brain damage.

Eye diseases associated with neovascularization also involve hypoxia. These eye diseases include diabetic retinopathy, retinopathy of prematurity, and sickle cell retinopathy. All can be serious enough to lead to blindness. The feasibility of treatment of retinopathy of prematurity by antisense inhibition of a hypoxia-induced gene, vascular endothelial growth factor (VEGF), has been demonstrated (Robinson, Patent No. 5,661,135).

A tissue associated with hypoxia-induced and hypoxia-repressed gene expression is the vasculature. There are four major cell types that comprise the vasculature, such as vascular endothelial cells, vascular smooth muscle cells, fibroblasts, and macrophages. The study of hypoxia-induced and hypoxia-repressed gene expression in these cell types *in vitro* and *in vivo* as a result of normal or pathophysiological conditions promises new insight into vascular diseases.

Hypoxia affects several mechanisms in cellular physiology, such as the transcriptionally regulated expression of vasoactive substances and matrix proteins involved in modulating vascular tone or remodeling the vasculature and surrounding tissue. Hypoxia results in the transcriptional induction of genes encoding vasoconstrictors and smooth muscle, and genes encoding matrix or remodeling molecules. Hypoxia also results in transcriptional inhibition of vasodilators such as endothelial nitric oxide synthase (eNOS) (Faller, D.V. (1999) *Clin. Exp. Pharmacol. Physiol.* 26(1):74-84).

The process of wound healing also involves the induction of gene expression by hypoxia (Anderson et al., Patent No. 5,681,706). TNF-α (tumor necrosis factor-α) expression and secretion by macrophages is one response involved in wound healing that is induced by low oxygen. Other hypoxia-induced effects include the formation of scar tissue.

In addition to playing a major regulatory role in the body's response to stress in postnatal life, tissue hypoxia is responsible for regulating expression of genes in the developing embryo, particularly with regard to angiogenesis and vasoformation (Iyer et al. (1998) *Genes and Development* 12:149-162; Maltepe et al. (1997) *Nature* 386:403-407). Hypoxia also plays a role in neonatal stress and pregnancy-related diseases. For instance, oxygen tension appears to regulate cytotrophoblast proliferation and differentiation within the uterus (Genbacev et al. (1997) *Science* 277:1669-1672). Some disease conditions related to pregnancy, such as preeclampsia, are associated with abnormal cytotrophoblast differentiation and behavior. A number of studies have shown that an increased concentration of a hypoxia-induced gene product, insulin-like Growth Binding Protein (IGFBP-1), is associated with preeclampsia once manifest in the third trimester, even though US Patent No. 5,712,103 teaches that reduced levels of IGFBP-1 in maternal blood in the first and second trimester, especially during the middle of the second trimester, can be used as a predictive indicator of preeclampsia.

Hypoxia has also been established to play a key role in neoplastic tissues. The progression of human tumors to malignancy is an evolutionary process involving the differential expression of multiple genes in response to unique microenvironments. Low oxygen conditions create a dominant tumor microenvironment which directly favors processes driving malignant progression, such as angiogenesis or elimination of p53 tumor suppressor activity.

In addition to promoting further tumor growth, the abnormally low oxygen levels that are found in nearly all solid tumors negatively impact therapeutic efforts. Hypoxic tumors often demonstrate resistance to radiation therapy and chemotherapy.

The connection between tumor hypoxia and the treatment of cancer is further exemplified by a study of cervical cancer that showed that the oxygen level of a tumor was an independent prognostic factor (Hoeckel et al. (1996) *Semin. Radiat. Oncol.* 6:1-8). The prognostic value of the oxygen level of a tumor was

found to be more significant than all other indicators such as the age of the patient, clinical stage, or tumor size.

A number of oxygen-regulated genes have been identified in the art. Expression of many of these genes is induced by the interaction of hypoxia inducible factor-1 (HIF-1), a transcription factor complex, with the factor's DNA recognition site on the gene, the hypoxia-responsive element (HRE). HIF-1 has been cloned and found to not be activated by stressors such as heat shock and ionizing radiation.

Differential-display polymerase chain reaction (PCR) has been used to identify additional genes induced by hypoxia (O'Rourke et al. (1996) *Eur. J. Biochem.* 241:403-410). Six hypoxia-induced genes were identified, three of which were of known function. In addition to the known genes, two expressed sequence tags (ESTs), and one full-length sequence were identified. The differential-display PCR method used by O'Rourke et al. to screen for hypoxically induced genes was found to be limited in its ability to identify hypoxically-induced genes.

In addition to the identification of hypoxia-induced and hypoxia-repressed genes, the identification of the stress-responsive regulatory elements of those genes is also of interest. The identification of such regulatory elements may provide for an inherently tumor-specific form of gene therapy. The HRE from a previously identified hypoxically induced gene, mouse phosphoglycerate kinase-1, has been used to control expression of heterologous genes both *in vitro* and *in vivo* (within a tumor) under hypoxic conditions (Dachs et al. (1997) Nature Medicine 3: 515-520). Similarly, a method for utilizing an anoxia-responsive element to effect controlled expression of a heterologous protein has been reported (Anderson et al., Patent No. 5,681,706).

SUMMARY OF THE INVENTION

The present invention relates to genes whose expression is modified under hypoxic conditions. The genes may be induced or repressed.

One aspect of the present invention provides the isolated polynucleotide having the sequence shown as SEQ ID NO:1 (Fig. 1A), comprising the cDNA of the hypoxia-induced human gene *HIG1,* and encoding the polypeptide sequence of SEQ ID NO:2 (HIG1; Fig. 1B). Polynucleotides with sequences complementary to SEQ ID NO:1, fragments of SEQ ID NO:1 which are at least twelve nucleotides in length, and sequences which hybridize to SEQ ID NO:1 are also contemplated by the present invention. In particular, one aspect of the invention concerns the fragment of the sequence set forth in SEQ ID NO:1 comprising nucleotides 62-343, the nucleotides representing the coding sequence of human *HIG1*. The complements to the coding sequence, at least twelve nucleotide-long fragments of the coding sequence, and sequences which hybridize to the coding sequence of *HIG1* are also provided by the invention.

Another aspect of the present invention provides the isolated polynucleotide having the sequence shown as SEQ ID NO:3 (Fig. 2A), comprising the cDNA of the hypoxia induced gene *HIG2,* and encoding the polypeptide sequence of SEQ ID NO:4 (HIG2; Fig. 2B). The complements to SEQ ID NO:3, as well as at least twelve nucleotide-long fragments thereof and sequences which hybridize thereto are also provided. The invention refers in particular to a polynucleotide having a sequence corresponding to nucleotides 274-465 of the sequence set forth in SEQ ID NO:3, or complements thereof, or at least twelve nucleotide-long fragments thereof, or sequences which hybridize thereto. Nucleotides 274-465 represent the coding sequence of human *HIG2*.

The present invention also encompasses expression vectors and delivery vehicles which contain polynucleotides of the present invention and host cells that are genetically engineered with polynucleotides of the present invention.

In another embodiment, the invention provides for an oligonucleotide probe comprising fragments, preferably at least

about 15 nucleotides long, of the polynucleotides of SEQ ID NO:1 or SEQ ID NO:3, or the complement thereto.

Polypeptides of the sequences set forth in SEQ ID NO:2 (HIG1) and SEQ ID NO:4 (HIG2), or biochemically equivalent fragments of the polypeptides of either sequence, are further contemplated by the present invention.

Antibodies that are specifically immunoreactive to the hypoxia-induced polypeptides HIG1 or HIG2 of the present invention are also provided.

In still another embodiment, the present invention provides for arrays of polynucleotides or polypeptides corresponding to at least two different hypoxia-inducible genes, hypoxia-induced polypeptides, or antibodies immunoreactive with hypoxia-induced polypeptides.

Hypoxia-inducible genes suitable for use in the arrays, diagnostic methods, and treatment methods of the invention described herein are not limited to *HIG1* and *HIG2*, or derivatives thereof, but also include a number of known genes now determined to be hypoxia-inducible. Additional hypoxia-induced genes useful in the methods and arrays of the present invention include, but are not limited to, the genes of *annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-1, endothelin-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular*

endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, insulin-like growth factor binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI1lDQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, angiogenin, and SDK3.

In one aspect, the present invention provides diagnostic and prognostic tools for assaying for the expression of hypoxia-inducible genes in a tissue of an animal, for determining the presence of hypoxia in a tissue in an animal, and for evaluating a hypoxia-related condition in an animal particularly in order to tailor therapy to a known hypoxic state. The detection of expression products, such as mRNA transcripts or proteins, of the hypoxia-inducible genes of *HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-1, endothelin-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster*

hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, insulin-like growth factor binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI1lDQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, angiogenin, or SDK3, or combinations thereof, to determine the presence of hypoxia in a tissue or evaluate a hypoxia-related condition in an animal is encompassed by the present invention. Methods of diagnosing and treating hypoxia-related conditions via such methods are also encompassed by the present invention.

Other methods of assaying for expression of hypoxia-inducible genes, determining the presence of hypoxia in a tissue in an animal, or evaluating a hypoxia-related condition in an animal involves the use of the arrays of the invention. First, a polynucleotide array or antibody array of the invention may be contacted with polynucleotides or polypeptides, respectively, either from or derived from a sample of body fluid or tissue obtained from the animal. Next, the amount and position of polynucleotide or polypeptide from the animal's sample which binds to the sites of the array is determined. Optionally, the

gene expression pattern observed may be correlated with an appropriate treatment.

In one aspect, the present invention provides for an expression array of polynucleotides to determine the presence of hypoxia in a tissue in an animal or a human, or to evaluate a hypoxia-related condition in an animal or a human. First, an expression array may be contacted with polynucleotides either purified or unpurified derived from a sample of body fluid or tissue obtained from the animal or human. Next, the amount and position of polynucleotide from the animal's sample which binds to the sites of the expression array is determined. Optionally, the gene expression pattern observed may be correlated with an appropriate treatment.

In a preferred embodiment of the invention, a gene chip (*vide infra*) may be contacted with polynucleotides either purified or unpurified derived from a sample of body fluid or tissue obtained from the animal or human. The amount and position of polynucleotide from the animal's sample which binds to the sites of the gene chip can then be determined. The gene expression pattern observed on the gene chip may be correlated with an appropriate treatment.

Another embodiment of the present invention provides for a diagnostic blood test for assaying for the expression of hypoxia-inducible genes in a tissue of an animal or human, and for detecting the presence of hypoxia-inducible gene products in a tissue in an animal or human. The detection of expression products, such as diagnostic marker proteins, of the hypoxia-inducible genes *of PAI-1, IGF-BP3, placental growth factor, adipophilin, mucin 1, endothelin-1, endothelin-2, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, EPH receptor ligand, angiogenin, TGF beta, HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-*

responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, brain HHCPA78, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI1lDQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, or SDK3, or combinations or derivatives thereof, to determine the presence of hypoxia in a tissue or evaluate a hypoxia-related condition in an animal or human is encompassed by the present invention.

Another aspect of the present invention provides for a diagnostic blood test for assaying for the expression of hypoxia-inducible genes in a tumor tissue of an animal or human, and for detecting the presence of hypoxia-inducible gene products in a tumor tissue in an animal or human. The detection of expression products, such as diagnostic marker proteins, of the hypoxia-inducible genes of *PAI-1, IGF-BP3, placental growth factor, adipophilin, mucin 1, endothelin-1, endothelin-2, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, EPH receptor ligand, angiogenin, TGF beta, HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen,*

phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, brain HHCPA78, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI11DQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, or SDK3, or combinations or derivatives thereof, to determine the presence of hypoxia in a tumor tissue or evaluate a hypoxia-related tumor condition in an animal or human is encompassed by the present invention.

In a preferred embodiment of the invention, the diagnostic marker proteins in the blood test are the hypoxia-inducible genes of *PAI-1, IGF-BP3, placental growth factor, adipophilin, mucin 1, endothelin-1, endothelin-2, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, EPH receptor ligand, angiogenin, or TGF beta.*

Another embodiment of the invention is a nuclear medicine based assay designed to non-invasively identify tumors of hypoxia *in vivo* by assaying for the expression of hypoxia-inducible genes in a tumor tissue of an animal or human, and by detecting the presence of hypoxia-inducible gene products in a tumor tissue in an animal or human. The detection of expression products, such as diagnostic cell surface ligands and receptors, of the hypoxia-inducible genes of *integrin alpha 5 receptor, interleukin-1 (IL-1) receptor, fibronectin, EPH receptor ligand, APO-1 (Fas Receptor), mucin-1, creatine transporter, monocarboxylate transporter*, or combinations or derivatives thereof, to determine the presence of hypoxia in a tumor tissue or evaluate a hypoxia-related tumor condition in an animal or human is encompassed by the present invention.

Other aspects of the invention concern treating a tissue which is a tumor by first determining the presence of hypoxia in the tumor and, second, treating the tumor with an established form of therapy for cancers such as radiation therapy, chemotherapy, and surgery.

In other aspects, the invention provides for methods of attenuating the hypoxic response of a tissue by blocking expression of a hypoxia-inducible gene *HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-1, endothelin-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer*

(BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, insulin-like growth factor binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cIIlDQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, angiogenin, or SDK3 in the cell or by neutralizing the polypeptide expression products of these genes in the tissue. The invention also provides for methods of treating hypoxia-related conditions by attenuating the hypoxic response of a tissue in an animal such as a human.

Methods for enhancing the response of tissue to hypoxia are provided in other embodiments of the present invention. These methods involve administering expression vectors comprising the hypoxia-inducible genes of the present invention or administering polypeptide expression products of hypoxia-inducible genes to the tissue.

Methods for identifying stress-inducible and stress repressible genes are also provided.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the human HIG1 cDNA and protein sequences. The nucleotide sequence for the human *HIG1* gene is shown in Figure 1A from 5′ to 3′(SEQ ID NO:1). The coding sequence is underlined.

The other regions are untranslated regions (5′ and 3′ UTR) of the gene. The protein sequence of human HIG1 is shown in Figure 1B (SEQ ID NO:2).

Figure 2 shows the human HIG2 cDNA and protein sequences. The nucleotide sequence for the human *HIG2* gene is shown in Figure 2A from 5′ to 3′ (SEQ ID NO:3). The coding sequence is underlined. The other regions are untranslated regions (5′ and 3′ UTR) of the gene. The protein sequence of human HIG2 is shown in Figure 2B (SEQ ID NO:4).

Figure 3 shows the murine HIG1 cDNA and protein sequences. The nucleotide sequence for the murine *HIG1* gene is shown in Figure 3A from 5′ to 3′ (SEQ ID NO:5). The coding sequence is underlined. The other regions are untranslated regions (5′ and 3′ UTR) of the gene. The protein sequence of murine HIG1 is shown in Figure 3B (SEQ ID NO:6).

Figure 4 shows the HIG1 cDNA and protein sequences of *seriola quinqueradiata*. The nucleotide sequence for this fish *HIG1* is shown in Figure 4A from 5′ to 3′ (SEQ ID NO:7). The coding sequence is underlined. The other regions are untranslated regions (5′ and 3′ UTR) of the gene. The protein sequence of fish HIG1 is shown in Figure 4B (SEQ ID NO:8).

Figure 5 shows the murine HIG2 cDNA and protein sequences. The nucleotide sequence for the murine *HIG2* gene is shown in Figure 5A from 5′ to 3′ (SEQ ID NO:9). The coding sequence is underlined. The other regions are untranslated regions of the gene (5′ and 3′ UTR). The protein sequence of murine HIG2 is shown in Figure 5B (SEQ ID NO:10).

Figure 6 shows the alignment of human HIG1 and HIG2 protein sequences with the HIG1 and HIG2 sequences of other species. The HIG1 homologues from humans (hHIG1), mice (mHIG1), and fish (*seriola quinqueradiate*) (fHIG1 or GHL1) are aligned in Figure 6A; the HIG2 homologues from humans (hHIG2) and mice (mHIG2) are aligned in figure 6B.

Figure 7 schematically illustrates the addition of linkers to cDNA library fragments. The linker addition is followed by PCR amplification.

Figure 8 illustrates how the subtraction protocol is used to enrich the tester cDNA library with sequences unique to the tester cDNAs.

DETAILED DESCRIPTION OF THE INVENTION

a) Definitions and General Parameters

The following definitions are set forth to illustrate and define the meaning and scope of the various terms used to describe the invention herein.

By the term "hypoxia" (or "hypoxic") is meant, for the purposes of the specification and claims, an environment of reduced oxygen tension such that the oxygen content is less than or equal to about 5%. In most cases, hypoxic tissue will have an oxygen content that is less than or equal to about 2%.

"Normoxic" or "oxic" conditions are conditions comprising a normal level of oxygen for that particular environment. Normoxic or oxic tissue typically has an oxygen content above about 5%.

The terms "hypoxia-induced" or "hypoxia-inducible" when referring to a gene means that the gene is expressed at a higher level when the host cell is exposed to hypoxic conditions than when exposed to normoxic conditions. Typically, the number of mRNA transcripts of a hypoxia-induced gene would is at least about 20% higher in a hypoxic cell versus a normoxic cell. Preferably, expression of the hypoxia-induced gene is at least about 2-fold higher in hypoxic versus normoxic cells. Most preferably, expression of the hypoxia-inducible gene is at least about 5-fold higher in hypoxic cells versus normoxic cells.

A "hypoxia-related condition" in an animal is a condition where hypoxia or altered (typically, enhanced) levels of expression of hypoxia-inducible genes in a tissue of the animal is involved. The hypoxia or altered expression of hypoxia-inducible genes may either be a symptom or play a role in the

cause, development, progression, amelioration, or cure of the condition. A hypoxia-related condition may optionally be a disease or pathological condition. Hypoxia-related conditions include, but are not limited to, cancer, ischemia, reperfusion, retinopathy, neonatal distress, preeclampsia, cardiac arrest, stroke, and wound healing.

The term "hypoxia-induced protein" or "hypoxia-induced gene product" means a protein encoded by a gene whose expression is induced by hypoxia.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, naturally-occurring polynucleotides or polypeptides present in a living animal are not isolated, but the same polynucleotides or polypeptides could be part of a vector or composition, and be isolated in that such vector or composition is not part of its natural environment.

A "sample obtained from a patient" or a "sample obtained from an animal" may be a sample of tissue or a sample of body fluid. The term "tissue" is used herein to refer to any biological matter made up of one cell, multiple cells, an agglomeration of cells, or an entire organ. The term tissue, as used herein, encompasses a cell or cells which can be either normal or abnormal (i.e. a tumor). A "body fluid" may be any liquid substance extracted, excreted, or secreted from an organism or a tissue of an organism. The body fluid need not necessarily contain cells. Body fluids of relevance to the present invention include, but are not limited to, whole blood, serum, plasma, urine, cerebral spinal fluid, tears, and amniotic fluid.

The term "biochemically equivalent variations" means protein or nucleic acid sequences which differ in some respect from the specific sequences disclosed herein, but nonetheless exhibit the same, or substantially the same, functionality. In the case of cDNA, for example, this means that modified sequences which contain other nucleic acids than those specifically disclosed are encompassed, provided that the alternate cDNA encodes mRNA which in turn encodes a protein of this invention. Such modifications may involve the substitution of only a few

bases, or many. The modifications may involve substitution of degenerate coding sequences or replacement of one coding sequence with another; introduction of non-natural nucleic acids is contemplated. It is not necessary for the alternate DNA to hybridize with that disclosed herein provided that the functional criterion is met. Preferably, the modified nucleic acid sequence hybridizes to and is at least 95% complementary to the sequence of interest.

Similarly, in the case of the proteins of this invention, alterations in the amino acid sequence which do not affect functionality may be made. Such variations may involve replacement of one amino acid with another, use of side chain modified or non-natural amino acids, and truncation. The skilled artisan will recognize which sites are most amenable to alteration without affecting the basic function.

A "polynucleotide", "oligonucleotide", or "nucleic acid" includes, but is not limited to, mRNA, cDNA, genomic DNA, and synthetic DNA and RNA sequences, comprising the natural nucleoside bases adenine, guanine, cytosine, thymine, and uracil. The term also encompasses sequences having one or more modified nucleosides. The terms "polynucleotide" and "oligonucleotide" are used interchangeably herein. No limitation as to length or to synthetic origin are suggested by the use of either of these terms herein.

The term "polypeptide" means a poly(amino acid) comprising at least two amino acids linked by peptide bonds. A "protein" is a polypeptide which is encoded by a gene.

"Neutralizing" a polypeptide or protein means inhibiting, partially or wholly, the bioactivity of the polypeptide or protein. This inhibition of activity may mean inhibition of catalytic activity, prevention of binding to a receptor or ligand, blockage or dimer formation, or the like.

The term "sequences which hybridize thereto" means polynucleotide sequences which are capable of forming Watson-Crick hydrogen bonds with another polynucleotide sequence under normal hybridization conditions, such as in buffered (pH. 7.0-7.5) aqueous, saline solutions (for instance, 1 to 500 mM NaCl) at room temperature. Although normal hybridization conditions

will depend on the length of the polynucleotides involved, typically they include the presence of at least one cation such as Na^+, K^+, Mg^{2+}, or Ca^{2+}, a near neutral pH, and temperatures less than 55°C. Although the sequences which hybridize to a polynucleotide may be about 90%-100% complementary to the polynucleotide, if the sequences are of sufficient length, in solutions with high salt concentrations, and/or under low temperature conditions, polynucleotides with complementarity of 70% or above, or even just 50% or above, may hybridize to the polynucleotide. Sequences which hybridize thereto typically comprise at least 15 nucleotides, and preferably at least about 30 nucleotides, which are complementary to the target polynucleotide.

A "coding sequence" is a polynucleotide or nucleic acid sequence which is transcribed and translated (in the case of DNA) or translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxy) terminus. A transcription termination sequence will usually be located 3′ to the coding sequence.

Nucleic acid "control sequences" refer to translational start and stop codons, promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, as necessary and sufficient for the transcription and translation of a given coding sequence in a defined host cell. Examples of control sequences suitable for eucaryotic cells are promoters, polyadenylation signals, and enhancers. All of these control sequences need not be present in a recombinant vector so long as those necessary and sufficient for the transcription and translation of the desired gene are present.

"Operably or operatively linked" refers to the configuration of the coding and control sequences so as to perform the desired function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. A coding sequence is operably

linked to or under the control of transcriptional regulatory regions in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA that can be translated into the encoded protein. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

The expression products described herein may consist of proteinaceous material having a defined chemical structure. However, the precise structure depends on a number of factors, particularly chemical modifications common to proteins. For example, since all proteins contain ionizable amino and carboxyl groups, the protein may be obtained in acidic or basic salt form, or in neutral form. The primary amino acid sequence may be derivatized using sugar molecules (glycosylation) or by other chemical derivatizations involving covalent or ionic attachment with, for example, lipids, phosphate, acetyl groups and the like, often occurring through association with saccharides. These modifications may occur *in vitro*, or *in vivo*, the latter being performed by a host cell through posttranslational processing systems. Such modifications may increase or decrease the biological activity of the molecule, and such chemically modified molecules are also intended to come within the scope of the invention.

"Vector" means a polynucleotide comprised of single strand, double strand, or circular DNA or RNA. An "expression vector" is comprised of the following elements operatively linked at appropriate distances for allowing functional gene expression: replication origin, promoter, enhancer, 5′ mRNA leader sequence, ribosomal binding site, nucleic acid cassette, termination and polyadenylation sites, and selectable marker sequences. One or more of these elements may be omitted in specific applications. The nucleic acid cassette can include a restriction site for insertion of the nucleic acid sequence to be expressed. In a functional vector the nucleic acid cassette contains the nucleic

acid sequence to be expressed including translation initiation and termination sites. An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences. Modification of the sequences encoding the particular protein of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; or to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site which is in reading frame with and under regulatory control of the control sequences.

A "regulatory element" is a segment of DNA to which a transcription factor(s) binds and alters the activity of a gene's promoter either positively (induction) or negatively (repression).

A "stress-responsive element" or "stress-responsive regulatory element" is a regulatory element which binds transcription factors activated by the cell in response to environmental stress. Environmental stressors may include one or more of the following: oxygen depletion; radiation; heat shock; pH change; hypothermia; or glucose starvation.

A "delivery vehicle", as used herein, refers to a means of delivering a polypeptide or a polynucleotide to a cell. The delivery vehicle is preferably used to deliver an expression vector to a cell or a cell in an organism. A delivery vehicle may be a virus, such as a retrovirus, an adenovirus, an adeno-associated virus, a herpes simplex virus, or a vaccinia virus.

Other possible delivery vehicles are non-viral. For instance, one of the many liposome formulations known to those skilled in the art, such as Lipofectin, may serve as a delivery vehicle. Liposomes are hollow spherical vesicles composed of

lipids arranged in a similar fashion as those lipids which make up the cell membrane. They have internal aqueous space useful for entrapping water soluble compounds such as polynucleotides. Recognition molecules can be attached to their surface for the targeting of the delivery vehicles to specific tissues.

As used herein, an "antibody" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. Antibodies may exist as intact immunoglobulins or as a number of fragments, including those well-characterized fragments produced by digestion with various peptidases. While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that antibody fragments may be synthesized de novo either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized de novo using recombinant DNA methodologies. Antibody fragments encompassed by the use of the term "antibodies" include, but are not limited to, Fab, Fab', $F(ab')_2$, scFv, Fv, dsFv diabody, and Fd fragments.

The phrase "specifically binds to a polypeptide" or "specifically immunoreactive with", when referring to an antibody refers to a binding reaction which is determinative of the presence of the polypeptide (or protein) in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to a protein under such conditions may require an antibody that is selected for its specificity for a particular protein or polypeptide. A variety of immunoassay formats may be used to select anitbodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are rountinely used to select monoclonal antibodies specifically immunoreactive with a protein.

b) Hypoxia-Inducible Genes and Expression Products

We have discovered a novel human gene, herein referred to as *HIG1*, whose expression is induced by cellular response to hypoxia (see the specific examples, Examples 1-6 below). We have isolated a cDNA of the human *HIG1* gene (SEQ ID NO:1; Fig. 1A) and identified the coding sequence to be nucleotides 62-343 of SEQ ID NO:1. The protein encoded by *HIG1* comprises the amino acid sequence shown in Figure 1B (SEQ ID NO:2). Polynucleotides with sequences complementary to SEQ ID NO:1, polynucleotides that are fragments of SEQ ID NO:1 of at least twelve nucleotides in length and polynucleotides which hybridize to SEQ ID NO:1 are also within the scope of the present invention. The fragments of SEQ ID NO:1 are preferably at least 15 nucleotides long.

In particular, polynucleotides comprising the nucleotides 62-343 of SEQ ID NO:1, or complements thereto, or at least twelve nucleotide long fragments thereof, or sequences which hybridize thereto are preferred. Fragments of the coding sequence of *HIG1* are preferably at least fifteen nucleotides in length.

We have also discovered a second, novel human gene, herein referred to as *HIG2*, whose expression is induced by cellular response to hypoxia. We have isolated a cDNA clone of this gene. The cDNA sequence of the *HIG2* gene is shown in Fig. 2A (SEQ ID NO:3). The coding sequence of *HIG2* comprises nucleotides 274-465 of SEQ ID NO:3. Fragments of the *HIG2* sequence, and of the *HIG2* coding sequence in particular, of at least twelve, and preferably fifteen, nucleotides in length are provided by the present invention as well. Polynucleotides of sequence which is complementary to SEQ ID NO:3 (especially to nucleotides 274-465) or polynucleotides which hybridize to polynucleotides of the sequence set forth in SEQ ID NO:3 (especially to nucleotides 274-465), are also contemplated.

Polypeptides encoded by the polynucleotides of *HIG1* (SEQ ID NO:2; Fig. 1B) and *HIG2* (SEQ ID NO:4; Fig. 2B), or biochemically equivalent variations of either protein, are also provided by the present invention. Fragments of these polypeptides which consist of at least eight amino acids are provided as well. Preferably, the fragments are at least 15 amino acids in length.

All biochemically equivalent variations of the aforementioned polynucleotides and polypeptides are considered to

be fully within the scope of this invention. The mouse and fish HIG1 polynucleotide and polypeptide sequences (Figs. 3, 4, and 6) can be considered biochemically equivalent variations of the human HIG1. The mouse HIG2 polynucleotide and polypeptide sequences (Figs. 5 and 6) are likewise understood to be biochemically equivalent variations of the human HIG1.

The polynucleotides of this invention may readily be incorporated within expression vectors by one of ordinary skill in the art. In a preferred embodiment, the polynucleotide sequence comprising nucleotides 62-343 of SEQ ID NO:1 (the coding sequence of HIG1) or nucleotides 274-465 of SEQ ID NO:2 (the coding sequence of HIG2) is operably linked with appropriate control sequences, such as a promoter.

Alternatively, larger fragments of the polynucleotides of SEQ ID NO:1 or SEQ ID NO:2 which comprise portions of the untranslated regions of the genes may be used in an expression vector instead. This may be particularly useful when hypoxia-inducibility is desired, since the untranslated regions may contain critical regulatory regions such as hypoxia-responsive elements.

The polynucleotides of this invention may also be incorporated within a host cell. In one embodiment, transfection may be used to introduce an expression vector containing one of the polynucleotides of the invention into the cell. The polynucleotide of the transfected vector may also be operably linked with control sequences including regulatory elements to effect the expression within the cell of exogenous protein or polypeptide sequences encoded by the polynucleotides of the present invention. Methods of cloning, amplification, expression, and purification will be apparent to the skilled artisan. Representative methods are disclosed in *Molecular Cloning: a Laboratory Manual, 2nd Ed., Vol. 1-3,* eds. Sambrook et al., Cold Spring Harbor Laboratory (1989).

A *HIG1* or *HIG2* polynucleotide may be introduced into an animal either by first incorporating the vector into a cell and then transferring the cell to the animal (*ex vivo*) or by incorporating the vector into a cell within an animal directly (*in vivo*).

The introduction of a *HIG1* or *HIG2* polynucleotide into a cell may be achieved by directly injecting the nucleic acid into the cell or by first mixing the nucleic acid with polylysine or cationic lipids which will help facilitate passage across the cell membrane. However, introduction of the polynucleotide into the cell is preferably achieved through the use of a delivery vehicle such as a liposome or a virus. Viruses which may be used to introduce a *HIG1* or *HIG2* polynucleotide or expression vector into a cell include, but are not limited to, retroviruses, adenoviruses, adeno-associated viruses, herpes simplex viruses, and vaccinia viruses.

Antisense oligonucleotides complementary to *HIG1* and *HIG2*, particularly those which are capable of blocking expression of *HIG1* or *HIG2* are provided by the present invention. The antisense oligonucleotide is preferably an oligonucleotide having a sequence complementary to at least a portion (preferably at least about 12 nucleotides in length) of SEQ ID NO:1 or SEQ ID NO:3. The antisense oligonucleotide is preferably between about 15 and about 22 nucleotides in length. Modifications of the sequence or bases of the antisense oligonucleotide may be desirable to facilitate transfer into a cell, stability, or tight binding to the *HIG1* or *HIG2* mRNA.

An oligonucleotide probe is provided by another embodiment of the invention. The probe consists of one of the polynucleotides of this invention, or an at least 12 nucleotide-long fragment thereof. The probe may be used to assay for, and if the probe is properly labeled, quantitate, the hypoxia-induced expression of *HIG1* or *HIG2* in a cell. In a preferred embodiment, the probe is at least about 15 nucleotides in length. In a particularly preferred embodiment, the probe is between 15 and 22 nucleotides in length.

Antibodies specifically immunoreactive with the HIG1 or HIG2 polypeptides represent still another embodiment of the invention. These antibodies may be monoclonal or polyclonal. The antibodies may optionally be recombinant or purely synthetic. The antibody may be an intact antibody or fragment. The preparation of antibodies specific to the HIG1 and HIG2 polypeptides would be routine for those skilled in the art.

In addition to the identification of the new genes HIG1 and HIG2 which were found to be hypoxia-inducible, we have also established for the first time that several previously known genes are hypoxia-inducible in humans (see the specific examples, Examples 2 and 9, below and Tables 6, 7, 8, and 9. These genes *annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-1, endothelin-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, insulin-like growth factor binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cIIlDQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2,*

carbonic anhydrase IX, TPI, angiogenin, and SDK3. Furthermore, expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), expression previously known to be hypoxia-inducible only in endothelial cells (Graven et al. (1998) Am. J. Physiol., 274(2 Pt 1):C347-355), is now shown by our work to be greatly induced in transformed cells. Additionally, a multitude of EST sequences from the databases have now been identified as being hypoxia-inducible (Table 3, Example 8, Table 5, Example 9, and Tables 7 and 8).

c) Polynucleotide, Polypeptide, and Antibody Arrays.

Another aspect of the invention involves the presentation of multiple (at least two, and preferably more than four) hypoxia-inducible gene sequences, polynucleotide probes complementary to the hypoxia-inducible gene sequences, hypoxia-induced polypeptides, or antibodies (immunoreactive with hypoxia-induced polypeptides) on an array. In particularly preferred arrays, more than about 10 different polynucleotides, polypeptides, or antibodies are presented on the array. In an alternative preferred embodiment, the number of different polynucleotides, proteins, or antibodies on the array is greater than about 25, even greater than about 100, or even greater than about 1000.

One aspect of the invention provides an array of polynucleotides which comprises at least two different hypoxia-inducible genes, or complements thereto, or at least twelve nucleotide-long fragments thereof, or sequences which hybridize thereto. The hypoxia-inducible genes or their fragments may optionally be selected from HIG1, HIG2, any of the hypoxia-inducible genes listed in Table 1 (below), Table 3 (Example 8, below), Table 5 (Example 9, below), and Tables 6, 7, 8, and 9. However, it is understood that all of the hypoxia-inducible gene sequences on the array need not be derived only from those hypoxia-inducible listed herein. The polynucleotides on the array are typically single-stranded.

For instance, in one embodiment of the polynucleotide array, on of the multiple polynucleotides on the array is derived from either the HIG1 or HIG2 gene sequences. The polynucleotides

of the array may comprise the entire sequence of one strand of the gene, or may comprise at least 12 nucleotide long fragments thereof, or sequences which hybridized thereto. In an alternative embodiment, one of the polynucleotides of the array comprises a polynucleotide corresponding to nucleotides 62-343 of SEQ ID NO:1 (*HIG1*) or nucleotides 274-465 of SEQ ID NO:2 (*HIG2*), or complements to one of the coding sequences, or at least twelve nucleotide-long fragments of one of the coding sequences, or sequences which hybridize to one of the coding sequences. In another embodiment of the polynucleotide array, at least one of the polynucleotide sequences of *HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-1, endothelin-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, insulin-like growth factor binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter*

isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI11DQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, angiogenin, and SDK3 is represented on the array in combination with a second, different polynucleotide sequence from a hypoxia-inducible gene. The second polynucleotide sequence may be selected from *HIG1*, *HIG2*, any of the hypoxia-inducible genes represented in Table 1 shown below, or Tables 6, 7, 8, or 9, any of the expressed sequence tags of hypoxia-inducible genes shown in Table 3 (see Example 8) or Tables 7 and 8, or any other hypoxia-inducible gene or expressed sequence tag from a hypoxia-inducible gene. Furthermore, the second polynucleotide sequence selected from any of the represented hypoxia-inducible genes can be derived from normal cells or tumor cells, exposed to hypoxic conditions. Table 7 ranks hypoxia-inducible genes by normal cell induction. Table 8 ranks hypoxia-inducible genes by tumor cell induction. Table 9 illustrates a hypoxic induction comparison of normal keratinocytes whereby genes are listed by increasing levels of hypoxic gene induction in normal dermal keratinocytes (NDK) and normal cervical keratinocytes (NCK). These are the normal cell counterparts of the cervical cancer cell lines (Siha and C33a), and the head and neck cancer cell lines (Fadu).

It is understood that regardless of which genes are represented on the array, the gene sequences do not have to be represented in their entirety.

The polynucleotide sequences that are immobilized on the array are most preferably, single-stranded and complementary to the mRNA transcripts of the relevant hypoxia-inducible genes. The immobilized polynucleotides may be fragments or complementary sequences of the gene or EST sequence that contain at least twelve nucleotides and preferably at least fifteen nucleotides. Alternatively, longer gene fragments including EST fragments of at least 50 or at least 100 nucleotides may be used. In a

preferred embodiment of the array, the array is made up of many different gene sequences.

In another embodiment of the polynucleotide array, only polynucleotides correlating to hypoxia-inducible genes expressing gene products of a similar function are included on the array. At least two, but preferentially more than two, different hypoxia-induced genes encoding proteins from a single functional category are represented on the array. Examples of eight functional categories of hypoxia-inducible proteins are as follows: (1) glycolytic enzymes/proteins; (2) angiogenesis/tissue remodeling proteins; (3) erythropoiesis/vascular regulatory proteins; (4) metabolic/homeostatic proteins; (5) apoptosis proteins; (6) DNA repair proteins; (7) cell-cycle proteins; and (8) transcriptional regulatory proteins. These categories are shown in Table 1, below, along with some representative members of each of the categories. It is understood that the members of each of the eight functional categories of hypoxia-inducible proteins are not limited to the lists shown in Table 1. It is further understood, that the list of functional categories of hypoxia-inducible genes is not limited to the eight categories listed in Table 1. Again, a preferred embodiment of this array comprises polynucleotide sequences complementary to the mRNA transcripts of the relevant hypoxia inducible genes. A particularly preferred embodiment of an array displays multiple polynucleotide sequences, each of which is complementary to a different gene which encodes a protein involved in angiogenesis and/or tissue remodeling.

Table 1. Eight Functional Categories of Hypoxia-Inducible Genes

GLYCOLYTIC ENZYMES/PROTEINS
lactate dehydrogenase (LDH)
phosphoglycerate kinase (PGK)
aldolase A
L-phosphofructokinase (PFKL)
glucose transporter isoform 3 (Glut-3)
interleukin-2
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
adenylate kinase isoenzyme 3 (AK-3)

ANGIOGENESIS/TISSUE REMODELING PROTEINS
vascular endothelial growth factor (VEGF)
placental growth factor
platelet-derived growth factor β (PDGFβ)
transforming growth factor β (TGFβ)
tumor necrosis factor α (TNFα)
interleukin-6 (IL-6)
interleukin-2 (IL-2)
tissue factor
fibroblast growth factor (FGF-3)
EPH receptor ligand
plasminogen activator inhibitor-1 (PAI-1)
macrophage migration inhibitory factor (MIF)
fibronectin receptor
lysyl hydroxylase-2
endothelin-2
integrin alpha 5
mucin 1
ERYTHROPOIEISIS/VASCULAR REGULATORY PROTEINS
erythropoietin (EPO)
tyrosine hydroxylase
heme oxygenase
alpha-fetoprotein (AFP)
endothelin
METABOLIC/HOMEOSTATIC PROTEINS
insulin-like growth factor binding protein-1 (IGFBP-1)
metallothionein
creatine kinase
inducible nitric oxide synthase (*i*-NOS-1)
epidermal growth factor receptor (EGFR)
huntingtin-associated protein 1 (HAP-1)
glucose-regulated protein 78 (GRP78)
glucose-regulated protein 90 (GRP90)
thioredoxin
annexin V
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1)
gamma-glutamyl cysteine synthetase heavy subunit
phosphoribosylpyrophosphate synthetase (PRPP synthetase)
acetoacetylCoA thiolase
fructose bisphosphatase

creatine transporter
fatty acid binding protein
glucose transporter isoform 3 (Glut-3)
adenylate kinase isoenzyme 3 (AK-3)
lactate dehyrogenase (LDH)
phosphofructokinase (PFK)
aldolase
lactate dehydrogenase (LDH)
glycogen branching enzyme
phsophoglycerate kinase 1 (PGK-1)
APOPTOSIS PROTEINS
insulin-like growth factor binding protein-3 (IGFBP-3)
c-myc
c-jun
Bcl-2-interacting killer (BIK)
19 kDa-interacting protein 3 long/Nip3-like protein X (NipP3L/Nix)
Pim-1
APO-1
DNA binding protein A20
DNA-REPAIR PROTEINS
Ku (70)
CELL-CYCLE PROTEINS
B-cell translocation gene-1 (BTG-1)
reducing agent and tunicamycin responsive protein (RTP)
CDC-like kinase-1 (clk-1)
quiescin (Q6)
growth arrest DNA damage-inducible protein 45 (GADD45)
GENE EXPRESSION AND TRANSCRIPTIONAL REGULATORY PROTEINS
RNAse L
differentiation of embryo chondrocytes (DEC1)
c-fos
Mxi-1

In an alternative embodiment of the polynucleotide array, polynucleotides correlating to the gene sequences encoding proteins belonging to at least two different functional categories of hypoxia-inducible genes are displayed on a single array. Although at least two different polynucleotide sequences are required to form the array, in a preferred embodiment many more than two are used. Again, a preferred embodiment of this

array comprises polynucleotide sequences complementary to the mRNA transcripts of the relevant hypoxia inducible genes of at least 12 nucleotides in length, and preferably fifteen.

The present invention also provides for polypeptide arrays analogous to the polynucleotide arrays discussed above, except that the polypeptide sequences of the hypoxia-inducible genes, or fragments thereof, are displayed in an array. The polypeptide array comprises the polypeptide expression products of at least two hypoxia-inducible genes, or biochemically equivalent fragments thereof. For instance, the polypeptide array my comprise the protein HIG1 or HIG2 and at least one other protein which is a hypoxia induced gene product. Alternatively, the polypeptide array may instead comprise at least one protein selected from the group consisting of HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-1, endothelin-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, insulin-like growth factor binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha,

interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI1lDQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, angiogenin, and SDK3, or a biochemically equivalent fragment thereof; and at least one of a second polypeptide which is a second hypoxia-induced gene product, or a biochemically equivalent fragment thereof.

Another aspect of the invention concerns a polypeptide array comprising at least two different hypoxia-induced proteins, or biochemically equivalent fragments thereof, wherein each hypoxia-induced protein belongs to a different functional category. Alternatively, the polypeptide array comprises at least two different hypoxia-induced proteins or biochemically equivalent fragments thereof, wherein said hypoxia-induced proteins are all proteins belonging to a single functional category. Optionally, the functional category may be selected from the group consisting of glycolytic enzymes/proteins, metabolic/homeostatic proteins, apoptosis proteins, DNA repair proteins, angiogenesis/tissue remodeling proteins, cell-cycle proteins, erythropoiesis/vascular regulatory proteins, and transcriptional regulatory proteins. (See Table 1, above.)

Yet another alternative embodiment of the invention, is an array analogous to a polypeptide array described above, except that antibodies immunoreactive with the hypoxia-induced polypeptides are immobilized to form the array, rather than the polypeptide sequences themselves. Each array comprises at least two different antibodies, each of which is immunoreactive with a different hypoxia-induced protein. Each of the two antibodies is specifically immunoreactive with the polypeptide expression products of hypoxia-inducible genes, such as, but not limited to, HIG1 or HIG2. For instance, in one embodiment, the antibody array comprises at least one antibody immunoreactive with a protein selected from the group consisting of HIG1, HIG2, annexin

V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-1, endothelin-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, insulin-like growth factor binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI1lDQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, angiogenin, and SDK3. The antibody array further comprises at least one of a second antibody, wherein said second antibody specifically binds a second hypoxia-induced gene product or a biochemically equivalent fragment thereof.

The antibodies on the array may be monoclonal or polyclonal. They may be intact antibodies or fragments of antibodies that are capable of specifically binding the polypeptides of the present invention. As is the case with the polynucleotide and polypeptide arrays of the invention, the antibody array preferably comprises at least four different antibodies, and preferably more than about 10 different antibodies.

Methods of constructing arrays of biomolecules, especially polynucleotides, have been previously established in the art. For instance, some methods for preparing particularly high density polynucleotide arrays are disclosed in Pirrung et al., Patent No. 5,143,854, Pirrung et al., Patent No. 5,405,783, Fodor et al., Patent No. 5,445,934, Fodor et al., Patent No. 5,510,270, Fodor et al., Patent No. 5,744,305, and Fodor et al., Patent No. 5,800,992, all of which are herein incorporated by reference. The polypeptides, antibodies, or polynucleotides may be immobilized on the array either covalently or noncovalently. Methods for immobilizing biomolecules are well known to those of ordinary skill in the art. The material to which the polynucleotides or polypeptides are immobilized in the array may vary. Possible substrates for construction of a biomolecule array include, but are not limited to, cellulose, glass, silicon, silicon oxide, silicon nitride, polystyrene, germanium, (poly)tetrafluorethylene, and gallium phosphide.

A gene expression array (gene chip) provides a quantitative method for monitoring and measuring hypoxia-related gene expression and may contain hundreds to thousands of genes and/or ESTs that are screened simultaneously. This allows for more rapid coverage of the genome. Once genes have been identified with the use of the gene expression array, their hypoxia inducibility and hypoxia repressability can be confirmed at the RNA level by Northern blotting or other techniques. There are many commercially available expression arrays such as the Atlas™ gene arrays (CLONETECH), GDA™ arrays and GEM™ microarrays (Incyte Pharmaceuticals, Inc.), the Affymetrix GeneChip®System (Affymetrix, Inc.), and others. However, expression arrays can also be produced directly with any number of genes and/or ESTs on

any number of materials, such as cellulose, glass, silicon, silicon oxide, silicon nitride, polystyrene, germanium, (poly)tetrafluorethylene, and gallium phosphide. For example, an array comprising from about 100 to about 1000 hypoxia-inducible and/or -repressible genes or more can be used to assess hypoxia-related conditions in animals and humans. The arrays can be carefully engineered to minimize non-specific hybridization with DNA or RNA probes. When hybridization is performed, the background levels are sufficiently low to permit detection of genes present at only few copies per cell. The sensitivity of the array permits the identification of genes that are expressed as low as only once per cell, which makes the array highly suitable to detect rare transcripts. An array comprising from about 100 to about 1000 hypoxia-repressible genes including, but not limited to, thrombospondin 1, stathmin, survivin, beta-tubulin or any of the genes or ESTs from Table 4 can be used to assess hypoxia-repression in animals and humans. Additionally, the small format and high density of the arrays not only permits the detection of rare transcripts but also the screening of many genes in parallel. This makes the use of expression arrays a valuable tool in research, diagnostics, and other pharmaceutical applications.

In one aspect, the present invention provides for an expression array of polynucleotides to determine the presence of hypoxia in a tissue in an animal or a human, or to evaluate a hypoxia-related condition in an animal or a human. First, an expression array may be contacted with polynucleotides either purified or unpurified derived from a sample of body fluid or tissue obtained from the animal or human. Next, the amount and position of polynucleotide from the animal's sample which binds to the sites of the expression array is determined. Optionally, the gene expression pattern observed may be correlated with an appropriate treatment.

In a preferred embodiment of the invention, a gene chip may be contacted with polynucleotides either purified or unpurified derived from a sample of body fluid or tissue obtained from the animal or human. The amount and position of polynucleotide from the animal's sample which binds to the sites of the gene chip can

then be determined. The gene expression pattern observed on the gene chip may be correlated with an appropriate treatment.

d) Methods of Use

In all of the methods of use described below, the animal is preferably a mammal. Most preferably, the mammal is a human.

We have demonstrated that the expression of *HIG1* or *HIG2* and a number of other genes is indicative of a cell's response to hypoxia as shown in the specific examples shown below (Examples 1-9). Accordingly, detection of abnormal levels of the transcripts of hypoxia-inducible genes such as *HIG1* or *HIG2*, or combinations thereof, in the tissues or body fluids of an animal can be used in both a diagnostic and prognostic manner for hypoxia-related conditions. The abnormal levels may be characterized by either increased levels or decreased levels, depending upon the hypoxia-related condition being analyzed. In other cases, either the complete absence or any presence of a hypoxia-inducible gene transcript may be indicative of an abnormal condition. Similarly, detection of abnormal levels of the hypoxia-induced polypeptides, or combinations thereof, can be used in either a diagnostic or prognostic manner for hypoxia-related conditions. The presence of hypoxia in a tissue can be evaluated by testing for the presence or absence of the transcripts or polypeptides encoded by the polynucleotides of the invention in either the tissue or in the body fluids of the animal. Detection of the transcripts or polypeptides can be either qualitative or quantitative.

One aspect of the invention, therefore, provides a method of determining the presence of hypoxia in a tissue in an animal or evaluating a hypoxia-related condition in a tissue in an animal. These methods comprise assaying for either the messenger RNA (mRNA) transcripts or the polypeptide expression product of at least one gene selected from the group consisting of *HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage*

migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-1, endothelin-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, insulin-like growth factor binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cIIlDQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, angiogenin, and SDK3 in a body fluid or the tissue of the animal. This method determining the presence of hypoxia in a tissue may be used to diagnose a hypoxia-related condition in a animal.

The presence of hypoxia in a tissue or the degree of expression of hypoxia-inducible genes determined by these methods may be used to select an appropriate treatment for the animal. For instance, the hypoxia-related condition being evaluated may be cancer and the tissue which is the target of the evaluation may optionally be a tumor. The degree to which the tumor is

showing gene expression patterns characteristic of hypoxia or the activation of genes involved in angiogenesis, for instance, can be usefully correlated with appropriate treatment of tumors of that particular type.

The hypoxia-related condition, however, need not necessarily be cancer. The hypoxia-related condition may instead be any condition in which hypoxic conditions play a role (favorable or detrimental to the animal). Such conditions include, but are not limited to, ischemia, reperfusion, retinopathy, neonatal distress, preeclampsia, cardiac arrest, stroke and wound healing.

The transcripts of hypoxia-inducible genes may be detected by any of several means known to those skilled in the art. One embodiment of diagnostic detection involves annealing to the transcript, *in vivo* or *in vitro*, a labeled nucleic acid probe complementary to the transcript sequence. The labeled probe can be fluorescent, radioactive, immunoreactive, colormetric or otherwise marked for detection. To detect very minute quantities of a transcript, amplification of the transcript in a tissue or fluid sample from the animal may first be performed to aid subsequent detection of the transcript. Amplification of the hypoxically-induced transcripts can be readily achieved using the polynucleotides of the present invention as primers, using reverse transcriptase to make a cDNA copy of the transcript, and then using polymerase chain reaction to achieve exponential amplification.

Detection of expression of the polypeptide products of the HIG1 or HIG2 genes, or any of the other hypoxia-induced genes could be achieved, for instance, by the application of labeled antibodies specifically immunoreactive with the polypeptide products. The antibodies can be applied to the tissue *in vivo*, or to tissue or body fluid samples removed from the animal. Various forms of typical immunoassays known to those skilled in the art would be applicable here. These assays include both competitive and non-competitive assays. For instance, in one type of assay sometimes referred to as a "sandwich assay", immobilized antibodies that specifically react with HIG2 polypeptide are contacted with the biological tissue or fluid

sample. Presence of the immobilized HIG2-antibody complex could then be achieved by application of a second, labeled antibody immunoreactive with either the HIG2 polypeptide or the HIG2-antibody complex. A Western blot type of assay could also be used in an alternative embodiment of the present invention.

If a removed tissue is to be analyzed *in vitro*, typically, degradation of the tissue is preferred prior to testing for the presence of either an mRNA transcript or a gene product. For instance, if detection of polynucleotides is desired, proteolytic degradation is useful (Temsamani et al., Patent No. 5,693,466). Extraction or isolation of proteins or nucleic acids in the sample is also preferred prior to carrying out a diagnostic screen. Numerous methods for the isolation of proteins or nucleic acids from cells or biological fluids are well established in the art.

In a preferred embodiment, a diagnostic evaluation of hypoxia-induced gene expression involves assaying the expression levels of more than one hypoxia-inducible inducible genes at a time. The arrays of the invention are particularly useful for assaying the expression of multiple hypoxia-inducible genes in parallel. The diagnostic detection methods mentioned above in regard to *in vitro* detection would also apply as methods for detecting the presence of polynucleotides and polypeptides in a tissue or a body fluid upon administration of a sample of the tissue or fluid to one of the arrays of the present invention.

Use of the polynucleotide or antibody arrays of the present invention for determining the presence of hypoxia in a tissue of an animal or for evaluating a hypoxia-related condition in a tissue of an animal allows for an unprecedented look at the exact nature and stage of the hypoxic response of a tissue, since the hypoxia-induced expression of a combination of genes is screened at one time. Patterns of expression of hypoxia-inducible (or hypoxia-repressible) genes are complex and highly indicative of hypoxia in a tissue, as demonstrated in the specific examples shown below, Examples 8 and 9. The pattern of expression of hypoxia-inducible genes can therefore be used in a diagnostic or prognostic manner to aid in the treatment of a hypoxia-related condition in an animal. Information on the pattern of expression

of a combination of hypoxia-induced genes can readily be correlated with the aggressiveness of a tumor for instance, thereby providing knowledge critical for establishing the best line of treatment.

The polypeptide arrays of the present invention also can be used to screen for drugs useful in the treatment of hypoxia-related conditions. These drugs may be drugs which are capable of inhibiting the hypoxic response of a tissue.

For instance, methods of assaying for expression of hypoxia-inducible genes in a tissue in an animal, determining the presence of hypoxia in a tissue in an animal, or evaluating a hypoxia-related condition in a tissue in an animal comprise first contacting the proteins or messenger RNA of a sample of body fluid or tissue obtained from the animal with an antibody array or polynucleotide array, respectively, of the invention. Tissue or fluid samples from an animal may be contacted directly with an array, and binding of the proteins or mRNA transcripts on the array detected. (The cells in a tissue to be assayed would preferably be lysed prior to application to the array.) Alternatively, the tissue or fluid sample may be purified to isolate the proteins or mRNA transcripts prior to application to the array. In an alternative embodiment of the method, cDNA is first prepared from the messenger RNA of the sample by reverse transcription and then the cDNA is applied to a polynucleotide array. Once the protein, mRNA or cDNA is delivered to the array, the method comprises detecting the amount and position of the protein, mRNA or cDNA which remains bound to the array after removal of excess or non-bound protein, mRNA, or cDNA.

Additionally, a method of diagnosing a hypoxia-related condition in an animal may optionally comprise the additional step of correlating the result of the evaluation of the hypoxia-related condition in the tissue in the animal with an appropriate treatment for the animal. The hypoxia-related condition which may be evaluated, diagnosed or treated by any of the above methods may a condition such as cancer, ischemia, reperfusion, retinopathy, neonatal distress, preeclampsia, cardiac arrest, or stroke.

Another aspect of the invention provides for a method of treating a tumor. This method involves first determining the presence of hypoxia in a tumor by any of the methods described above (with or without arrays). The method further comprises treating said tumor with any combination of an established form of therapy for cancer such as radiation therapy, chemotherapy, or surgery.

The *HIG1* or *HIG2* polynucleotides or the polynucleotides corresponding to the gene sequences of other hypoxia-inducible gene sequences, such as those listed in Table 1, may be used to attenuate the response of a tissue to hypoxia. These hypoxia-inducible sequences can be targeted within a tissue by the introduction of antisense oligonucleotides, triple-helix probes, catalytic nucleic acids or the like in a manner which inhibits expression of the HIG genes or other hypoxia-inducible genes within the tissue.

Therefore, in one embodiment, the method of attenuating the hypoxic response of tissue comprises inhibiting the expression of a gene selected from the group consisting of *HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-1, endothelin-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, insulin-like growth factor*

binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI1lDQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, and SDK3 in said cell. This inhibition of expression of a hypoxia-inducible gene may optionally be achieved by introducing into the cells of said tissue a nucleic acid molecule such as an antisense oligonucleotide, a triple-helix probe, a deoxyribozyme, or a ribozyme which is specific to the hypoxia-inducible gene.

In an alternative embodiment of the invention, the HIG1 or HIG2 proteins or other expression products of hypoxia-inducible genes may instead be targeted to attenuate the hypoxic response of a tissue. For this purpose, antibodies, antagonists, inhibitors, or proteases that are specific to the expression products of hypoxia-induced genes may be introduced to the tissue.

In one embodiment, a method of attenuating the hypoxic response of a tissue comprises neutralizing a protein selected from the group consisting of HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-1, endothelin-2, B-cell translocation

gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, insulin-like growth factor binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI1lDQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, angiogenin, and SDK3. In this method an agent specifically targeting the protein is optionally introduced into the cells of the tissue and can be an antibody, an antagonist, an inhibitor, or a protease.

The methods described above for attenuating the hypoxic response of a tissue may be used to treat a hypoxia-related condition in an animal. For instance, the treatment of a hypoxia-related condition in an animal may be effected by targeting the hypoxia-induced gene sequences of the hypoxic (or potentially hypoxic) tissue via one or more of the techniques known to those skilled in the art. These techniques include, but are not limited to, introduction of antisense oligonucleotides, triple-helix probes, deoxyribozymes, or ribozymes into the

subject's tissue of concern. In a preferred embodiment, the animal to be treated is a human. The hypoxia-related condition towards which this treatment may be directed is ischemia, stroke, heart attack, neonatal distress, retinopathy, or any other disease condition in which hypoxia plays a significant role. In another embodiment, the hypoxia-related condition to be treated is cancer and the tissue is a tumor. The disclosed treatment of the tumor may be coupled with any combination of other cancer therapies such as radiation therapy, chemotherapy, or surgery.

Similarly, treatment of the hypoxia-related conditions may also be achieved by neutralizing the protein expression products of hypoxia-inducible genes, as described above. In accordance with this method, antibodies, antagonists, inhibitors, proteases, or the like which target and neutralize HIG1 and HIG2 polypeptides may be introduced into the animal, preferably human, containing the tissue to be treated.

The protein expression products of the genes which have been newly identified as being hypoxia-inducible may be used to identify or screen for drugs, such as inhibitors, useful in the treatment of hypoxia-related conditions. For instance, small molecule drug candidates or peptides may be tested against the any of the proteins of HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-1, endothelin-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular

endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, insulin-like growth factor binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cIIlDQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, angiogenin, or SDK3 to see if inactivation of the enzymatic activity or prevention of crucial binding activity of the hypoxia-induced protein occurs. Combinatorial libraries of small molecules or libraries of peptides such as those produced by phage display may alternatively be screen against one of the hypoxia-induced proteins described herein.

The expression of some gene products induced by hypoxia can be helpful in protecting cells from damage or death. Thus, this invention also provides for methods of enhancing the hypoxic response of a tissue and thereby and treating hypoxic tissue (or potentially hypoxic tissue). The method comprises introducing an expression vector into the tissue and allowing for expression of the coding sequence on the vector to take place. The coding sequence of the expression vector comprises the sequence of at least one of the genes *HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-1, endothelin-2, B-cell translocation*

gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, insulin-like growth factor binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI11DQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, angiogenin, or SDK3. Expression of the vector's hypoxia-inducible gene within the tissue should occur at a level which is higher than would occur in the absence of the expression vector. Depending on the particular use, the coding sequence of the expression vector may be operably linked to its native promoter, another hypoxia-inducible promoter, or a constitutive promoter.

Alternatively, the proteins of the hypoxia-inducible genes may be introduced into the tissue directly to enhance the hypoxic response of the tissue and for treatment of hypoxia. Delivery of the proteins may be achieved through the use of liposomes, hydrogels, controlled-release polymers, or any of the other

vehicles known in the art to be useful for the delivery of polypeptides as drugs.

e) Methods for Identifying Stress-Inducible Genes

To facilitate efforts to identify hypoxia-inducible genes, we modified and improved a PCR subtraction method known as Representational Difference Analysis (RDA) (see specific example, Example 1, below, and Figures 7 and 8). The RDA method has been used to distinguish differences between genomic DNA from two related, but different sources (Wigler et al., Patent No. 5,436,142). The RDA technique involves selectively amplifying via polymerase chain reaction only fragments of those sequences contained within one DNA sample, but not the other. The selectivity of the amplification step used in this method is not precise, but is sufficient to detect differences in the genomes of two human individuals.

The present invention provides for methods of identifying both stress-inducible and stress-repressible genes. The methods identify differences between mRNA from cell populations exposed to different stress conditions. A representative protocol for the identification of stress-inducible genes is outlined in detail in a specific example below (Example 1).

The method for identifying stress-inducible or stress-repressible genes and fragments of genes involves first subjecting one of two populations of cells to stress prior to preparation of two cDNA libraries from the mRNA libraries of the two populations. Protocols for the generation of cDNA libraries through reverse transcription of mRNA sequences are well known in the art and kits for doing so are commercially available (from Gibco BRL, for instance). In a preferred embodiment of the method, the cDNAs are synthesized by using a mixture of oligo-dT primers containing equal proportions of oligomers having a G, A, or C residue at the 3′-end ("indexed" or "registered" primers). This approach ensures that a given primer will hybridize at the start of a polyA tail sequence of an mRNA rather than randomly within the sequence. These oligo-dT primers also have a defined DNA sequence (20 to 24 base pairs in length) that is incorporated into each cDNA fragment. This tag permits the use of two PCR

primers to specifically amplify the 3'-end of each cDNA. The two cDNA libraries are digested separately with restriction enzymes and then linker sequences are ligated to the ends of the digested cDNA fragments, as shown in Fig. 7. Restriction digests and ligation of linkers may be performed in any manner known to those skilled in the art. Some examples of such methods may be found in Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual, 2nd. ed*, Cold Spring Harbor Laboratory Press, herein incorporated by reference.

The cDNA library from one of the two cell populations is amplified with tagged oligonucleotide primers by means of the polymerase chain reaction (PCR). In a preferred embodiment, the "tag" on the oligonucleotide primers is biotin. However, any chemical or biological moiety which provides a means of selection or isolation of the tagged entity (by affinity chromatography, for instance) is suitable as a tag. In the preferred embodiment, use of biotin as a tag allows for removal of the tagged sequences on a streptavidin resin. In an alternative embodiment, however, oligonucleotides bearing a thiol group, for example, may instead be used as the tagged primer, since oligonucleotides with attached thiol groups can be retained on a variety of affinity resins, such as thiopropyl sepharose columns or mercurial resins. The cDNA library PCR-amplified with tagged primers is referred to herein as "driver" cDNA.

The cDNA library from the stressed cells is amplified with normal, non-tagged, oligonucleotide primers in a separate polymerase chain reaction. The cDNA PCR-amplified in this manner is referred to herein as "tester" cDNA.

The non-tagged, amplified, tester cDNA is heated and then reannealed in the presence of a large excess (typically about 5- to about 100-fold) of the tagged, amplified, driver cDNA. See Fig. 8. Next, those DNA strands which either are themselves tagged or are duplexed with tagged DNA are removed from the mixture. This removal is typically done via exposure of the mixture of DNA strands to a resin or matrix which has affinity for the tag used on the primers earlier. In a preferred embodiment, magnetic beads coated with streptavidin are used. Other resins, such as streptavidin agarose could be used in

conjunction with a biotin tag. Tagged single-stranded or duplex cDNA will be retained on the affinity resin, and the non-tagged species, which are not retained, can be found in the flowthrough or supernatant. In this technique, the cDNA from the non-stressed cell population is "subtracted" from the cDNA of the stressed cell population. The remaining, non-tagged cDNA library is said to be "enriched". The remaining, non-tagged cDNA sequences are then again amplified by means of the polymerase chain reaction with non-tagged primers.

After amplification of the remaining non-tagged cDNA sequences, the non-tagged cDNA library is again heated and reannealed in the presence of a large excess (typically about 5- to about 100-fold) of the original tagged cDNA library. Removal of all tagged DNA molecules and reamplification of remaining tagged sequences again follows. The combination of steps involving heating and reannealing, removed tagged molecules, and reamplifying remaining, non-tagged molecules constitutes one round. The methods of the present invention involve repeating the rounds from zero to many times. In a preferred embodiment, the method involves a total of approximately 3 to 5 rounds.

In a particularly preferred embodiment, the method involves performing the steps as described above in parallel with a second set of steps in which the cDNA library from the stressed population of cells is instead subtracted from the cDNA library from the non-stressed population. This means that in the second set of steps, the cDNA library from the stressed cell population is amplified with tagged primers and the cDNA library from the non-stressed cell population is amplified with non-tagged primers. The original cDNA of the stressed cell population is repeatedly subtracted from the cDNA of the non-stressed cell population, and separately, the original cDNA of the non-stressed cell population is repeatedly subtracted from the stressed cell population.

In the final round of the preferred embodiment of the method, one of the two enriched cDNA libraries obtained from the two sets of steps is subtracted from the other enriched cDNA library. Which enriched library is subtracted from which is entirely dependent upon whether stress-inducible or stress-

repressible sequences are sought. If stress-inducible sequences are sought, the enriched, non-stressed cDNA library is subtracted from the enriched, stressed, cDNA library. If stress-repressible sequences are sought, the enriched, stressed-cell cDNA library is subtracted from the enriched non-stressed-cell cDNA library.

The final subtraction step of one enriched library against another is beneficial since the initial subtraction rounds of the procedure tend to remove only the cDNAs that are in common and present at high frequency in the two populations, because cDNA fragments derived from rare messages will initially be present at such low concentrations that they might not find a complementary strand during the hybridization step. After the major sequences in common are removed by subtraction, the rare sequences will begin to increase in concentration so that they can then be effectively subtracted. After multiple cycles of subtraction are performed, the rarest sequences from both conditions are enriched in the libraries, and subtraction of one enriched library from another yields an effective isolation of either stress-inducible or stress-repressible genes.

After the desired number of rounds of subtraction have been completed, the enriched cDNA library may be cloned and sequenced using any one of the multitude of techniques known to those skilled in the art. A particularly convenient method of inserting PCR-amplified DNA strands into vectors suitable for cloning and sequencing, known as "T-A cloning", is commercially available from companies such as Invitrogen and Novagen. Other alternative methods can be found in *Molecular Cloning: A Laboratory Manual, 2nd. ed, Vol. 1-3,* eds. Sambrook et al., Cold Spring Harbor Laboratory Press (1989).

In one embodiment, the stress to which one of the two cell populations is exposed is hypoxia. The method may also be applied to the investigation of responses to other stresses, such as ionizing radiation, heat, glucose starvation, hypothermia, or pH change. Alternatively, the response to a stress such as a toxin or a drug may be investigated by employment of the disclosed method.

f) Diagnostic Blood Test Using Hypoxia-Inducible Marker Gene Products

A new diagnostic blood test, disclosed herein, allows for the detection of hypoxia-related conditions. Hypoxia-responsive genes produce marker gene products that can be measured in the blood stream of humans and animals. A blood test has been devised to test for these diagnostic marker gene products whereby secreted proteins are the basis for measuring tumor hypoxia (see Example 10 below). Secreted proteins can be measured in the bloodstream of humans or animals with solid tumors. The oxygen status of each tumor sample is determined through independent measurement techniques including, but not limited to, a nitroimidazole-binding technique (EF5) or the Eppendorf oxygen electrode. These applications can determine if there is a relationship between the oxygen status and blood levels of the hypoxia marker gene. Serum levels of secreted marker proteins are assayed through commercially available ELISA kits that are well known in the art. Serum levels can also be assayed through proteomic techniques, immunohistochemistry, immune blotting, and other techniques that are well known in the art.

One aspect of the present invention provides for a diagnostic blood test for assaying for the expression of hypoxia-inducible genes in a tissue of an animal or human, and for detecting the presence of hypoxia-inducible gene products in a tissue in an animal or human. The detection of expression products, such as diagnostic marker proteins, of the hypoxia-inducible genes *of PAI-1, IGF-BP3, placental growth factor, adipophilin, mucin 1, endothelin-1, endothelin-2, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, EPH receptor ligand, angiogenin, TGF beta, HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin,*

growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, brain HHCPA78, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI1lDQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, or SDK3, or combinations or derivatives thereof, to determine the presence of hypoxia in a tissue or evaluate a hypoxia-related condition in an animal or human is encompassed by the present invention.

A further aspect of the present invention provides for a diagnostic blood test for assaying for the expression of hypoxia-inducible genes in a tumor tissue of an animal or human, and for detecting the presence of hypoxia-inducible gene products in a tumor tissue in an animal or human. The detection of expression products, such as diagnostic marker proteins, of the hypoxia-inducible genes of *PAI-1, IGF-BP3, placental growth factor, adipophilin, mucin 1, endothelin-1, endothelin-2, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, EPH receptor ligand, angiogenin, TGF beta, HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase,*

ribosomal L7, fibroblast growth factor-3 (FGF-3), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, brain HHCPA78, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI11DQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, or SDK3, or combinations or derivatives thereof, to determine the presence of hypoxia in a tumor tissue or evaluate a hypoxia-related tumor condition in an animal or human is encompassed by the present invention.

In a preferred embodiment of the invention, the diagnostic marker proteins used in the blood test are the hypoxia-inducible genes of *PAI-1, IGF-BP3, placental growth factor, adipophilin, mucin 1, endothelin-1, endothelin-2, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, EPH receptor ligand, angiogenin, or TGF beta.*

g) Nuclear Medicine Used To Identify Hypoxia-Related Conditions Through Surface Markers

One possible avenue to assess conditions related to tumors with hypoxia *in vivo*, is the use of nuclear medicine based assays designed to non-invasively identify tumors. Tumor hypoxic regions can be detected through the non-invasive imaging of the cell surface using nuclear medicine approaches such as Magnetic Resonance Imaging (MRI), Nuclear Magnetic Resonance (NMR), or others well known in the art. Imaging reagents that assist in the detection of tumor regions may be adminstered intravenously or orally. Cell surface ligands and receptors such as integrin alpha 5 and the interleukin-1 (IL-1) receptor are good targets for this type of nuclear medicine based imaging of hypoxia. These gene products are good candidates because the are localized to the tumor areas where they are expressed on the cell surface, and they are accessible to systemically administered imaging reagents.

One embodiment of the invention is a nuclear medicine based assay designed to non-invasively identify tumors of hypoxia *in vivo* by assaying for the expression of hypoxia-inducible genes in a tumor tissue of an animal or human, and by detecting the presence of hypoxia-inducible gene products in a tumor tissue in an animal or human. The detection of expression products, such as diagnostic cell surface ligands and receptors, of the hypoxia-inducible genes of *integrin alpha5 receptor, interleukin-1 (IL-1) receptor, fibronectin, EPH receptor ligand, APO-1 (Fas Receptor), mucin-1, creatine transporter, monocarboxylate transporter,* or combinations or derivatives thereof, to determine the presence of hypoxia in a tumor tissue or evaluate a hypoxia-related tumor condition in an animal or human is encompassed by the present invention.

h) Examples

The following specific examples are intended to illustrate the invention and should not be construed as limiting the scope of the claims.

Example 1. Generation of Enrichment cDNA Libraries

Normal human cervical epithelial cells stably immortalized with the human papillomavirus E6 and E7 oncoproteins (HCE.E6.E7) served as the starting material for the construction of a cDNA library enriched by representational difference analysis (RDA). HCE.E6E7 were cultured in synthetic medium PFMR-4A (Kim et al. (1997) *Cancer Res.* 57:4200-4).

A total of 5 µg of poly A^+ mRNA from both HCE.E6E7 cells cultured under hypoxic (5% CO_2/5% H_2/90% N_2 for 16 hours at 37°C) conditions and HCE.E6E7 cells cultured under aerobic (5% CO_2 / 20% O_2 / 75% N_2 at 37°C) conditions were used to generate double-stranded cDNA preparations by using the Gibco BRL cDNA Synthesis System.

Hypoxic conditions were generated by the use of an anaerobic chamber (Sheldon Laboratories, Cornelius OR) that is flushed with a gas mixture of 90% N_2, 5% CO_2 and 5% H_2. Any oxygen that was introduced into the chamber was consumed over a catalyst with hydrogen. A monitoring oxygen electrode was used to confirm an environment of 0.05% oxygen or less during experimentation.

One-fifth of the cDNA product (approximately 1-1.5 µg) from the hypoxic or oxic cells was digested with 20 units of the *Nla* III restriction enzyme, 50 mM potassium acetate, 1 mM DTT, and 100 µg/ml bovine serum albumin for 60 min at 37°C. The reaction mixture was extracted with phenol and chloroform, precipitated with ethanol, redissolved in 10uL of water and lyophilized. Ethidium agarose gel electrophoresis was used to verify that the cleavage was successful.

For each pair of cDNAs used for the RDA procedure (i.e. the "test" and the "driver"), two different DNA linkers were attached by ligation to the *Nla* III cleaved ends. The 3'-end of the linker sequence opposite the ligation site was terminated with an amine so that it cannot be used as an acceptor or donor for a ligase. The linker oligonucleotides used were as follows (where "X" denotes the animo-terminated residue at the 3'-end of the shorter of the two strands):

5′-TTTTACCAGCTTATTCAATTCGGTCCTCTCGCACAGGATGCATG-3′ (SEQ ID NO:11)
XATGGTCGAATAAGTTAAGCCAGGAGAGCGTGTCCTAC-5′ (SEQ ID NO:12)

5′-TTTTTGTAGACATTCTAGTATCTCGTCAAGTCGGAAGGATGCATG-3′ (SEQ ID NO:13)
XAACATCTGTAAGATCATAGAGCAGTTCAGCCTTCCTAC-5′ (SEQ ID NO:14)

(The linker pair of SEQ ID NO:13 and SEQ ID NO:14 was used for the hypoxically incubated cell cDNAs.) The two separate linker strands were dissolved in 10 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$ buffer (10 µM of each oligomer), then heat-denatured and slowly cooled to room temperature before use in a ligation reaction.

Next, 1 µg of the *Nla* III cleaved cDNA was ligated in a 100 µL volume of 1 µM double-stranded linker, 5% polyethylene glycol, 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, and 1 mM DTT at 16°C for 3 h. Since the linkers used to ligate to the cDNA fragments do not have a phosphate at the 3′-end of the *Nla* III overhang, the resulting ligation products have a single-stranded nick. Performing the reaction in this way had the advantage of preventing self-ligation of the linkers. The excess linkers were removed by gel filtration through a spin-column containing Sephacryl S-300HR. The linker-ligated cDNA fragments were collected in the microfuge tube while the excess unligated linkers were trapped in the Sephacryl with other low molecular-weight components. The gel-filtered, linker-ligated cDNA fragments were then lyophilized to dryness.

The linker-ligated cDNA fragments were amplified by a single-primer PCR technique. Again, if the preparation was to be used as the driver cDNA, it was amplified by using PCR primers with a biotin residue at the 5′-end. If the preparation was to be used as the test cDNA from which the driver is used to subtract sequences, then it was amplified by using untagged primers.

The ligated cDNA (0.1 µg aliquot) was amplified in a standard PCR buffer containing 1 µM primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin. Before PCR amplification, the nicked PCR template had to be repaired by TAQ polymerase during a 5-min extension reaction at 72°C. After this

initial incubation, a standard PCR reaction of 35 cycles (94°C, 30s; 56°C, 30s; 72°C, 60s) was performed in a Perkin Elmer DNA Thermal Cycler. The oligonucleotide primers used in the amplification step were as follows:

5'-CCAGCTTATTCAATTCGGTCC-3' (SEQ ID NO:15)
5'-GTAGACATTCTAGTATCTCGT-3' (SEQ ID NO:16)

(SEQ ID NO:16 was the primer used to amplify cDNA from the hypoxically incubated cells.) The entire PCR reaction was passed through a 1 ml Sephacryl spin column as described above to remove salts, dNTPs, and excess primers. The yield of the amplification was determined by ethidium agarose gel electrophoresis. The product appeared as expected as a smear of DNA fragments ranging from 100 to 2,000 base pairs (bp) in size.

The first round of subtraction was performed by mixing 3 μg of the biotinylated driver cDNA with 0.1 μg of the test cDNA. The mixture was lyophilized in a 0.5 mL microfuge tube and carefully redissolved in 2 μL of 50 mM HEPES (pH 7.5), 10 mM EDTA, 1.5 mM NaCl, and 2% sodium dodecyl sulfate (SDS). This very small amount of solution was overlaid with 50 μL of mineral oil to prevent evaporation, and the tube was place in the thermal cycler and heated at 95°C for 10 min. It was then slowly cooled to 68°C over a period of 1 h, after which the incubation at 68°C was continued for a further 4 hours. At the end of the incubation, 100 μl of the same HEPES buffer at 68°C was added to the tube. The diluted solution was then cooled to room temperature and the mineral oil removed.

The biotinlyated cDNAs and any hybridized sequences were removed by mixing the diluted solution with a 100 μL slurry containing 1 mg of M-280 Streptavidin Dynabeads (Dynal) in the same incubation buffer. The incubation was continued at room temperature for 30 min with slow tumbling. The beads were then pelleted to the bottom of the tube by using a magnet and the supernatant was removed and desalted by passing through a 1 mL Sephacryl spin column as described above. The cDNA solution was then lyophilized and redissolved in 10 μL of water.

The small amount of cDNA remaining after subtraction was reamplified by PCR using the same primers. A single-stranded binding protein was added to the PCR reaction mixture used to reamplify the subtracted cDNA fragments: 1 µL (one-tenth volume) of the subtracted cDNA preparation was placed in 100 µL of PCR buffer containing 1 µg of *Escherchia Coli* single-stranded binding protein (Perfect Match™, Stratagene). The cDNA was amplified during 25 PCR cycles (94°C, 30 s; 54°C, 30 s; 72°C, 60 s), and the product was analyzed by ethidium agarose gel electrophoresis. The appearance of this reamplified cDNA was similar to that of the initial material described above.

Multiple rounds of subtraction were performed. The subtraction libraries were prepared in parallel, so that the library enriched for sequences expressed under hypoxic conditions was prepared at the same time as the library enriched for sequences expressed under normoxic conditions. In each case, the driver used for the initial rounds of subtraction was the original set of cDNA fragments. After three rounds of subtraction, the enriched library prepared in parallel was used as the driver for the fourth round. In this way, the rarest sequences from both conditions were enriched in the final library. For instance, to obtain hypoxically induced sequences in this final round, the cDNA library enriched for sequences expressed under normoxic conditions served as the driver library and the cDNA library enriched for sequences expressed under hypoxic conditions served as the test library.

Example 2. Sequence Identification and Northern Blot Analysis of Significant Isolated Expressed Sequence Tags (ESTs).

Several hundred cDNA fragments were sequenced from each of the two enrichment libraries produced by the subtraction protocol of Example 1 from HCE.E6E7 cells cultured under hypoxic and aerobic conditions. Four rounds of RDA subtraction of the oxic cDNAs from the hypoxic cDNAs generated a population of fragments in one of the enrichment libraries representing genes that theoretically are induced by hypoxic treatment. Five hundred randomly chosen clones from the cDNA library were partially sequenced. The obtained sequences were analyzed by NCBI-blast to

determine the frequency of each of the genes/ESTs in the enriched population and to identify whether the isolated, hypoxia-induced ESTs corresponded to previously identified genes or ESTs.

The frequencies of EST sequences among the 500 randomly chosen cDNA fragments obtained from the cDNA library enriched for sequences expressed under hypoxic conditions (after all four rounds of subtraction) is shown in Table 2, below. The two most frequently occurring ESTs, the HIG1 EST and the HIG2 EST, corresponded to no known genes. Because these most frequently repeated clones were unknown, the full-length cDNAs representing HIG1 and HIG2 were isolated (see Example 3, below)..

All the ESTs present in the clones of each library that were represented more than one time and that did not contain a highly repetitive element were tested by Northern blot for induction by hypoxia in Siha cervical carcinoma cells (and/or in HCE.E6E7 cells). Selected probes representing ESTs found more than once were applied to Northern blots of total RNA from cell cultures harvested following different aerobic and hypoxic exposures to verify hypoxia inducibility or repressibility. For instance, the northern blot assays were used to confirm that, α-tubulin mRNA, detected in the HCE.E6E7 aerobic enrichment library, decreased in response to hypoxia in HCE.E6E7 cells, whereas mRNA corresponding to the HIG2 EST, found in the hypoxic enrichment library, strongly increased under the same hypoxic conditions.

Table 2. Tags isolated from the cDNA library following four rounds of RDA subtraction of oxic cDNAs from hypoxic cDNAs.

# of hits	tag name	gene to which EST corresponds	accession # of gene	response to hypoxia*	comment
106	HIG1	HIG1		induced	novel gene
98	HIG2	HIG2		induced	novel gene
48	HIG3	GAPDH	J04038	induced	inducibility prev. known
11	HIG4	HNRNP	X12671	induced	
11	HIG5	Annexin V	U01691	induced	
8	HIG6	AcetoacetylCoA thiolase	S70154	induced**	
7	HIG7	Tissue Factor	X67698	induced	inducibility

					prev. known
7		5-2A bp	X76388	not induced	
6		unknown gene	clone 68	no signal	
5		Alu-like		not determined	
5	HIG8	Lipocortin 2	M14043	induced	
5	HIG9	Ribosomal L7	X57959	induced	
4		unknown gene	clone 24	not induced	
3		Vacuolar ATPase	X71490	no signal	
3	HIG10	PRPP synthase	D00860	induced	
3		Alu-like		not determined	
2		RNA pol1 40Kd subunit	AF047441	not induced	
2	HIG11	thioredoxin	X77584	induced	inducibility prev. known
2		hSRP1(nuc loc)	U28386	not induced	
2	HIG12	Ku(70)	J04611	induced	
2		Sm protein F	X85382	not induced	
1		168 different clones			

* as determined by Northern blot

** minor 4.2kB acetoacylCoA thiolase message only is induced

The procedure for the Northern blot assay was essentially as follows. Total RNA was isolated with Trizol (Gibco BRL) following the directions of the manufacturer. 5-10 µg of total RNA was denatured with glyoxal and size fractionated on a 1% agarose phosphate gel. The gel was capillary transferred to Hybond nylon (Schleicher and Shuell) and UV cross-linked. Probes were radiolabeled by random priming of gel-purified full length HIG1, or a fragment of HIG2 containing only the coding sequence in a Stu1 fragment (Rediprime, Amersham). Hybridization was carried out in 0.5 M Na_2HPO_4, 7% SDS, 1 mM EDTA at 56°C for HIG1 and 65°C for HIG2, washed to 0.2-0.5 x SSC at 56°C or 65°C, exposed to a phosphorimager plate, and visualized on a Storm 860 phosphoimager (Molecular Dynamics).

The hypoxia-inducibility of ESTs as determined by Northern blot is summarized in Table 2, above. The HIG1 and HIG2

sequences both demonstrated hypoxia-inducibility in the Northern blot assay.

Northern blots of total RNA from various aerobic and hypoxic human cells [HCE.E6E7s; SiHa cervical squamous carcinoma, MCF-7 breast carcinoma, H1299 lung carcinoma, Hct116 colonic carcinoma cells; human cervical fibroblasts (HCFs) and HCF.E6E7s] probed for HIG2 expression demonstrated the following: (1) the gene is expressed as a single 1.5 kb transcript (the original EST cross-hybridizes with unknown 1.6- and 4-kb transcripts in HCE.E6E7s); (2) HIG2 mRNA increases from undetectable in 21% O_2 (air) to abundant in 0.02% O_2 in HCE.E6E7, SiHa, and MCF-7 cells after 6 h of hypoxia; (3) HIG2 is moderately expressed in H1299 and Hct116 cells after 6 h of hypoxia; (4) there is no detectable HIG2 mRNA in HCFs and HCF.E6E7s; (5) in SiHa cells, HIG2 remains elevated for 48 h of hypoxia but decreases moderately by 72 h of exposure; and (6) no HIG2 induction is found in SiHa cells 6 h and 24 h after treatment with UV-C (20 J/m^2), γ-irradiation (6 Gy), MMS (100 $\mu g/mL$ for 1 h), serum deprivation (0.1%), or glucose starvation (4%, <1 mM); (7) HIG2 expression is extinguished after exposure of hypoxic cells to 2 hours of reoxygenation.

The hypoxia inducibility of HIG1 has been found to range between about 2-fold and about 5-fold across a variety of different human cell lines studied. The hypoxia-inducibility of HIG2 ranges between about 10- and about 20-fold across the various human cell lines studied. (See also Example 4, below).

In addition to the novel genes *HIG1* and *HIG2*, several known genes identified by the subtraction method in Example 1 were confirmed by Northern blots to be hypoxia inducible. These genes are also listed in Tables 2, 6, 7, 8, and 9. ESTs corresponding to the genes of annexin V, lipocortin 2, hnRNP A1, Ku (70) autoantigen, glyceraldehyde-3-phosphate dehydrogenase, ribosomal L7, acetoacetylCoA thiolase, and PRPP synthetase were identified by multiple hits in the hypoxia screen. All of these previously known genes were confirmed to be hypoxia-inducible by Northern blot.

It should be noted that although acetoacetyl CoA thiolase sequence tag is listed as induced, the reported, major RNA (1.8

kb) for the gene does not change. However, there is a larger, hybridizing, RNA species (4.2 kb) that is induced after 24-48 h hypoxia (data not shown).

ESTs corresponding to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were especially prevalent amongst the cDNA clones. The hypoxia-induced expression of glyceraldehyde-3-phosphate dehydrogenase had been previously identified only in normal, non-transformed cells.

Example 3. Isolation and Analysis of Full-Length *HIG*1 and *HIG2* cDNA Sequence

The HIG2 EST (142 bp) was used to probe a conventional cDNA library constructed from mRNA isolated from SiHa cells exposed to 16 h hypoxia to obtain the full-length cDNA clone *HIG2*. This library was probed with radiolabelled HIG2 tag using conventional methods. Full length *HIG1* was isolated by first identifying overlapping ESTs from the NCBI human EST database, until a full length sequence was generated (1.35 kb). PCR primers were then synthesized corresponding 5′ and 3′ UTRs in order to amplify the complete sequence using RT-PCR of SiHa RNA isolated after a 16 h hypoxia treatment. The full-length *HIG1* cDNA was then cloned and sequenced to confirm the predicted sequence.

The full-length cDNA sequence of *HIG1* is shown in Figure 1A. The full-length cDNA sequence of *HIG2* is shown in Figure 2A. The translations of the putative open reading frames from *HIG1* and *HIG2* are listed in Figure 1B and 2B, respectively, and both encode small peptides (95 and 64 aa residues respectively) without obvious functional motifs.

Example 4. Hypoxic induction of *HIG1* and *HIG2* in cervical cancer cell lines.

Because *HIG1* and *HIG2* represent two novel genes whose functions are unknown, these genes were investigated in more detail. The expression of *HIG1* and *HIG2* was examined in a series of human cervical cancer cell lines (SiHa, CaSki and C33a) under oxic and hypoxic conditions *in vitro*. (The cell lines SiHa, CaSki and C33a were obtained from the ATCC and were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI1640

supplemented with 10% fetal bovine serum.) Although *HIG1* is induced moderately within 2 hours of hypoxia in all the cell lines tested, it remains elevated only in the Siha cells. *HIG2* is more consistently induced from low basal levels in all the cervical cancer cells tested. The major *HIG2* mRNA species is 1.4 kb in length, but there are two other mRNA species of minor abundance (8.0 and 9.0 kb) that are induced with identical kinetics to the major species.

Example 5. Hypoxic induction of *HIG1* and *HIG2* in tumor xenografts.

The hypoxic induction of *HIG1* and *HIG2 in vivo* was also tested in tumor xenografts generated from the C33a cell line by Northern blot analysis of total tumor RNA. Gene expression in untreated xenografts was compared to that in xenografts that were made hypoxic by treatment of the host animal with flavone acetic acid (FAA) 24 hours prior to explantation and RNA isolation. To generate tumor xenografts, 2.5-5 x 10^6 cells were injected subcutaneously into the flank of scid mice and allowed to grow into tumors that reached 1-2 cm in diameter before harvest. FAA (Lipha Chemical, NY) was injected IP into the animals at 200 mg/kg in 5% sodium bicarbonate 24 hours prior to tumor harvest. FAA treatment resulted in increased tumor hypoxia as measured by ependorff electrode and increased *HIG1* and *HIG2* expression by 1.2 and 2.4 fold respectively. The moderate level of *HIG1* induction *in vivo* is not unexpected, due to the *in vitro* data. The portion of the human gene used for a probe in these experiments has low homology with mouse RNA and under the conditions used, did not cross-hybridize.

Example 6. Specificity of the induction of *HIG1* and *HIG2*.

We next investigated whether HIG1 and *HIG2* induction is unique to hypoxic stress, or if it is elicited by other tumor microenvironment stresses such as glucose deprivation, serum starvation, or by genotoxic stresses such as UV or ionizing radiation. We also tested the hypoxia-mimetic, iron-chelating compound desferoxamine that has been shown to induce expression from HIF-1 responsive genes. For stress treatments, cells were

plated overnight and then treated the next day with either 256 nm UV at 1.2 J/m^2/sec, or gamma irradiation from ^{137}Cs source at 3.8 Gy/min. Glucose and serum deprivation experiments were performed by washing the cells three times in phosphate-buffered saline (PBS) and replacing the indicated media (glucose free RPMI with dialyzed serum, or 0.1% FBS RPMI).

Northern blot analyses was performed on RNA isolated from C33a cells exposed to these stresses. *HIG1* was poorly responsive to hypoxic stress over this time course, but strongly induced by glucose deprivation. *HIG2* was induced strongly by hypoxia, the hypoxia-mimetic stress desferoxamine (DFO), and glucose deprivation. UV light seemed to have little effect upon either *HIG1* or *HIG2* expression. In contrast, while ionizing radiation did not change *HIG1* expression levels, it did result in a moderate 2.5 fold induction of *HIG2* by 24 hours. There were similarities in the pattern of stress responsiveness of *HIG2* and that of the HIF-responsive VEGF gene, suggesting that HIF-1 may be important in *HIG2* expression.

Example 7. Identification of *HIG1* and *HIG2* sequences from non-human species.

A search of the NCBI-dbEST database for fragments of genes from other species that might represent evolutionarily conserved orthologues identified overlapping mouse EST fragments that encode for similar peptides to the human version of *HIG1* and *HIG2*. The murine *HIG1* and *HIG2* orthologues are shown in Figures 3A and 5A, respectively. These mouse genes code for predicted peptides (Figures 3B amd 5B, respectively) with 84% and 76% identity to the human peptides respectively. There also existed a cDNA cloned from fish (*seriola quinqueradiata*) in the database that coded for a HIG1 orthologue (Figure 4A and 4B). A sequence comparison of the HIG1 homologues is shown in Figure 6A. A sequence comparison of the HIG2 homologues is shown in Figure 6B.

We confirmed the existence of murine HIG1 and HIG2 by cloning the presumed genes and assaying for their expression. We designed oligonucleotide primers corresponding to sequences in the 5′ and 3′ untranslated regions that would amplify these genes. We were able to make primers that amplified the entire

murine *HIG2* cDNA, but were only able to make primers that would amplify the coding sequence for murine *HIG1*:

mHIG1 forward primer (SEQ ID NO:17):
5'-CCGATCTAGAGGAAGGGACCCCGCGTCTCGGA-3'
mHIG1 reverse primer (SEQ ID NO:18):
5'-GGCGCTCGAGTCTAAACCCACATGTTATTTATTG-3'
mHIG2 forward primer (SEQ ID NO:19):
5'-CCTTACTCCTGCACGACCTGG-3'
mHIG2 reverse primer (SEQ ID NO:20):
5'-GGCGCTCGAGCACATGTGCATTACACTGGAGA-3'

These primers were then used to amplify the coding sequences of *HIG1* OR *HIG2* from reverse-transcribed RNA isolated from the murine squamous cell tumor cell line SCCVII (cultured in DMEM supplemented with 10% FBS). The amplified fragments were cloned and sequenced, confirming the predicted sequence.

The cloned genes were then used as probes for Northern blot analysis of RNA isolated from SCCVII cells. Both m*HIG1* (murine *HIG1*) and m*HIG2* (murine *HIG2*) have hypoxia-inducible species of RNA by this analysis. Murine *HIG1* has two major RNA species that strongly hybridize to the probe, at approximately 1.2-1.4 kb in length. The larger message is modestly induced, while the smaller message is strongly induced to approximately 5 fold by a 12h exposure to hypoxia. Murine *HIG2* also has two RNA species at approximately 1.4 and 2.2 kb. Both the murine *HIG2* mRNAs seem to be mildly hypoxia-inducible with 2-3 fold induction by 6-12 hours. For comparison, the same blot was probed with vascular endothelial growth factor (VEGF) and this message shows an approximately 5-fold induction by 6h.

Example 8. Analysis of Gene Expression under Hypoxia using Gene Discovery Arrays (GDA).

Nylon filters containing GDA arrays were purchased from Genome Systems (St Louis, MO) that have affixed to them nucleic acids that were originally characterized by the I.M.A.G.E. consortium (LLNL). This array represents 18,394 cDNA clones that have been categorized as either known genes or ESTs (expressed sequence tags) isolated by the consortium. This filter was used

to quantitatively determine the mRNA expression levels of all these arrayed cDNAs in SIHA tumor cells both under oxic conditions and hypoxic conditions (18 hrs, <0.2 %). Messenger RNA was isolated from control and hypoxic SIHA cells and cDNA probe was generated using MoML reverse transcriptase. 2 µg mRNA was incubated with 500 ng of oligonucleotide primer $(T)_{18}$ NM (N=A/C/G, M=A/C/G/T) in the presence of reaction buffer, 4mM dATP, 4mM dGTP, 4 mM dTTP and 4mM alpha $^{[33]}$P dCTP and 200U reverse transcriptase. The radioactively labeled first strand cDNA that was produced from this reaction was then used to probe the respective filter. The filters were then exposed to a phosphoimager plate, the image collected and digitized for analysis, and the relative counts on each cDNA were quantitated and compared using GDA analysis software. The results are shown in Table 3 for the 500 genes or ESTs with the greatest level of hypoxic induction and in Table 4 for the 500 genes or ESTs with the greatest level of hypoxic repression.

Table 3. Genes (identified by Genbank Accession Number) whose expression was induced in hypoxic cells, shown with the ratio of their expression in hypoxic cells over their expression in oxic cells.

ratio	Acc. No.	ratio	Acc. No.	ratio	Acc. No.	ratio	Acc. No.
5.18	AA069408	7.013	AA134027	4.027	AA155910	3.372	AA037436
7.528	T48883	5.236	N35559	5.343	T87461	2.025	H22698
9.999	N58711	5.753	R63553	3.478	W24548	5.454	W07146
5.678	H04904	6.525	N27733	5.699	T48772	5.209	AA101069
6.453	H82707	3.146	N94304	4.095	N94916	4.599	W24109
7.825	W56465	4.218	H70730	6.52	H14897	4.996	R17409
9.999	N31409	1.659	AA053856	1.159	R34659	4.932	W17090
9.999	H19264	8.836	W05763	2.492	H93923	5.391	R24601
3.626	R73213	3.394	R54524	5.054	N28535	5.189	R26954
9.999	R08251	8.246	W15599	2.08	AA069499	2.12	AA187216
9.999	H91612	4.216	R09918	5.021	W38635	3.852	H86677
9.999	AA196038	9.238	AA005185	8.59	T54127	4.653	H67329
9.999	W55913	4.654	T95404	4.289	R26319	4.993	W19173
5.143	R94248	9.262	R19946	6.048	W07082	5.641	W06851
9.999	R85589	4.308	AA068998	3.12	AA194330	6.186	W00378
2.9	H50204	5.203	N47831	4.594	N31417	4.458	AA204792
4.333	H52973	4.175	AA151009	2.424	AA069173	4.64	W48584
9.999	H60510	5.33	W46682	3.645	W52472	3.742	R84764
9.915	R13129	5.39	AA176700	6.109	H09049	4.049	R21449
9.999	AA040826	3.612	H47207	3.843	H81775	5.074	AA160325
2.186	N76562	7.666	W87527	5.664	H71710	2.356	R97269
8.468	R13125	5.965	W47502	2.494	W17237	3.68	H86214
9.999	W52400	6.338	N44758	3.516	H06318	4.654	N40829
3.376	AA054303	2.313	AA126937	4.006	T63499	5.114	W19928
4.263	AA042800	6.232	R31353	5.829	R22383	5.526	H18298
2.681	W46165	4.846	R18798	3.457	T87920	2.867	N39173
8.287	W24084	4.648	T78246	5.766	W47525	3.251	R76943
9.999	T77247	6.726	H29713	5.493	W32575	4.139	W07720
8.506	R13073	4.524	AA026304	5.622	R84635	3.604	AA085920
9.999	T95699	6.113	AA053162	5.284	R18138	3.814	H18258
8.7	N30952	1.702	H65775	3.414	R34648	3.192	AA035131
8.372	N29065	3.685	W32710	4.46	H80175	2.275	AA033736
7.37	H13744	6.264	T51305	4.479	R76163	3.896	R71065
9.999	H46657	7.182	R14403	4.381	W17182	3.534	AA074177
6.115	H83517	3.9	W38235	5.81	AA126956	4.358	R92264
6.587	AA129780	5	W47083	3.781	AA088390	3.561	N34634
6.587	N42542	5.209	W35243	4.048	R52046	3.695	AA114861
9.999	H67546	3.288	W24343	5.474	N98916	4.673	R31970
6.678	AA181350	5.209	H93373	3.226	R51929	3.989	H70974
7.187	T67190	6.255	R26331	3.033	H69334	4.646	AA156193

ratio	Acc. No.	ratio	Acc. No.	ratio	Acc. No.	ratio	Acc. No.
6.498	AA034932	2.549	R70082	5.458	W20511	3.258	AA085385
9.999	H41372	4.698	W32969	3.742	T40473	4.453	W49770
9.999	H08885	4.038	R66920	5.132	R82723	4.056	W37672
8.221	H90627	6.586	W25455	5.886	T50788	2.503	W38097
4.812	T91423	7.516	W38478	3.266	AA036758	5.118	N48838
5.535	N49031	1.961	AA039447	3.564	W46219	5.105	H41937
7.673	AA079020	3.163	H89835	3.535	N64406	1.544	AA159807
5.718	H44892	3.311	AA156148	4.839	W17076	3.436	AA112478
2.058	H38180	5.592	W38424	5.314	H18129	4.396	R07212
3.101	T48613	6.954	H75477	4.118	H93835	4.917	AA128281
3.236	H12952	3.507	W31707	5.306	AA167017	4.303	N39630
2.553	T87585	3.968	N40606	5.012	W24939	4.165	W39234
2.961	R76842	3.417	W67757	3.887	W38826	1.983	H68587
4.505	N54105	5.57	AA074760	3.791	H62991	3.358	H78277
3.424	W72986	2.311	H18350	4.853	AA132801	2.968	T87597
3.738	H53662	2.707	W31757	2.065	R54128	3.142	H64978
1.682	W05551	3.252	T89571	3.35	AA214334	4.376	W16557
2.942	H62026	3.202	W07148	3.553	W16484	3.316	H45068
3.16	AA146611	2.404	H66389	3.902	AA122157	3.559	H08997
3.702	H70850	3.621	R68331	3.451	H44677	3.277	H08983
4.118	W49687	4.089	AA099075	3.277	W20192	2.455	H66256
4.359	AA100113	4.46	H91361	3.534	T47067	2.632	T70457
3.338	AA133312	2.057	N42428	3.581	T82048	2.569	H13942
4.367	T49117	3.423	W48763	3.694	AA134135	2.59	T77584
3.79	H58461	3.844	R60387	2.79	R25979	2.346	R71543
4.806	N69323	3.421	W19744	1.586	R21064	3.168	T98705
2.664	W05089	2.473	R59435	1.972	H29698	3.43	T78542
3.176	AA070823	3.189	R07238	3.507	R89708	2.887	T74951
1.966	W24455	2.496	AA074340	3.189	R06568	3.768	W16974
3.258	R63252	3.287	N78038	1.957	AA009869	3.48	R88098
4.543	H80571	3.935	W07144	1.627	W31940	2.021	W48791
3.593	AA131550	4.133	R06175	3.545	W38638	3.686	R80458
4.34	N42413	3.912	N46036	1.634	R60752	1.812	H29706
2.313	R62688	2.535	W78057	2.917	R28459	3.501	AA130339
2.952	H73881	3.614	R16609	3.614	H63610	2.642	H10811
5.245	AA007484	3.208	H01260	3.568	R66182	2.323	AA074067
6.009	N57562	1.818	W04913	2.77	H95908	2.419	T56791
3.62	H25971	3.591	R61631	2.83	H24644	2.432	R48720
3.583	H19106	3.528	H71668	2.458	AA044130	2.881	R20222
3.085	W46660	4.66	R18905	2.039	R23341	3.274	H03764
3.694	R36401	2.311	H64449	3.785	H01679	2.71	R55247
3.945	R09905	1.619	W07452	2.82	T91461	3.324	AA005419
4.416	H40081	3.462	AA203284	3.202	T74959	2.494	T97640
4.498	AA156956	4.843	N66473	1.924	T39976	4.157	R23556
3.22	AA112421	3.777	H78279	3.135	H68817	3.956	N53743
2.991	AA147722	3.256	AA156298	3.739	H83559	1.947	W01963

ratio	Acc. No.	ratio	Acc. No.	ratio	Acc. No.	ratio	Acc. No.
4.341	W20171	3.25	N75101	1.841	T79362	3.858	N94762
1.778	W21312	3.961	H75277	2.551	N36269	3.655	AA007521
3.42	N28517	4.366	R80450	3.285	T79536	1.506	R00903
3.436	W07026	3.532	AA057729	3.193	T87507	2.775	H83982
4.393	R21898	2.373	T92805	3.87	T71354	2.61	H12686
3.543	R36586	3.167	AA005286	3.572	W19860	2.577	H78353
2.784	T48691	3.33	H12796	2.896	AA039258	3.227	N45476
4.06	W16946	1.679	R60831	4.245	T79534	2.799	R55692
3.071	T85481	3.64	T85390	2.096	R59467	3.196	W31182
4.116	T83199	2.606	N73091	3.168	H91401	2.371	T91436
2.479	H19169	2.648	T56084	2.074	W24718	2.951	T54610
3.768	W37172	3.4	W37084	2.318	T54602	2.735	AA001324
2.146	H70732	3.531	W07043	2.636	H65057	4.007	H02412
4.475	N75228	2.86	N44537	3.254	R52482	3.547	N92284
3.428	W00630	3.546	T74332	2.377	H95979	2.257	H78485
2.951	N57398	2.2	R35560	2.4	R80523	3.216	R20594
3.62	W15521	3.945	R69162	2.953	H79197	3.416	H12419
3.262	R61036	2.337	T48694	3.711	R85335	1.522	R91771
2.572	T68568	3.228	N31889	2.546	T56622	3.225	H09280
1.5	N40660	2.72	R60420	3.107	AA205009	2.957	H63806
2.193	N98743	3.234	AA004897	2.83	W00950	2.588	R70441
3.464	W16685	2.893	R24648	2.271	N73209	3.093	R19326
2.372	T82120	2.187	AA063234	2.596	N31231		
2.765	T61346	3.766	R96692	2.953	T85879		
4.054	AA214079	3.217	AA004891	2.655	R32750		
1.542	AA164677	3.915	W00391	1.922	N32733		
2.937	AA211776	3.291	R82770	3.495	W17002		
3.067	T87472	4.545	H18766	2.984	W20484		
3.058	H61812	3.167	R87818	1.377	AA136789		
2.937	N45602	2.214	W88806	2.364	H45241		
2.927	R14907	3.318	T54086	1.678	W17311		
3.476	H12508	2.992	H61280	3.688	R87413		
3.25	W19104	3.13	AA167039	3.432	R14301		
2.441	R87193	3.287	T87358	2.927	R80475		
2.521	H92713	2.711	W03009	3.258	T79546		
2.617	R32216	2.674	N98348	3.18	T78497		
2.262	AA126184	3.311	N47460	1.595	R52015		
3.281	H09869	2.187	H73438				
2.02	AA054041	2.751	W78830				
2.639	H66558	2.953	T49575				
3.709	R07731	3.122	N47660				
3.032	T80874	3.435	H03226				
3.673	H51373	3.371	H42536				
3.114	T51726	3.049	T85153				
3.137	H09884	2.421	N53255				
3.202	T98755	2.379	R70814				

ratio	Acc. No.	ratio	Acc. No.	ratio	Acc. No.	ratio	Acc. No.
3.201	AA194172	2.33	W47650				
1.981	H13009	3.072	T78498				

Table 4. Genes (identified by Genbank Accession Number) whose expression was repressed in hypoxic cells, shown with the ratio of their expression in oxic cells over their expression in hypoxic cells.

ratio	Acc. No.	ratio	Acc. No.	ratio	Acc. No.	ratio	Acc. No.
9.999	H38055	7.873	N33752	4.55	R59009	9.999	R31317
6.948	AA057425	6.351	T54424	3.78	AA121166	6.082	AA057398
9.999	AA116099	8.097	T87470	8.936	R01823	6.504	R89643
9.999	AA057428	7.137	H06343	7.628	AA085375	7.172	H70359
9.999	R73197	4.887	AA058878	3.185	AA054115	5.273	N48132
9.999	R48415	9.999	H46055	8.052	H96006	4.543	AA054096
9.999	H14999	6.651	T40066	5.324	T67226	5.207	AA054071
9.999	T96535	9.672	R89521	5.895	R62231	4.911	AA078915
9.999	H27140	9.999	AA065190	4.767	AA112466	4.439	H52742
9.999	T99054	7.455	H43837	4.271	N34169	3.092	T67415
9.999	H46382	4.601	W81199	5.635	H51983	3.457	R49895
9.999	R90757	9.999	H27344	6.176	N52679	4.953	H16042
9.999	AA121402	9.999	H49310	6.465	H50403	6.039	H45590
9.999	H38676	9.999	R69813	4.812	H26200	6.767	AA029349
7.52	AA057511	9.999	N32666	6.495	N30528	6.456	H51981
9.999	N43944	2.046	AA053873	7.015	H47146	5.186	R61165
9.999	AA134018	9.999	AA125970	5.891	R11999	2.541	R85183
5.741	H14566	4.712	H83338	5.442	N24303	4.764	H85692
9.999	AA035019	9.999	R94457	3.989	H14332	5.288	DROS-A8
9.285	N29018	4.545	N31674	6.206	R64420	4.603	R24904
7.813	AA069149	5.6	T50828	6.203	R37898	3.307	W72875
9.999	R76214	7.861	R74161	6.251	R76298	4.033	H71729
9.999	R23999	7.437	T99984	5.33	N98261	6.431	H14193
9.999	R23880	5.5	R48041	6.206	R24405	8.731	N48042
9.999	AA078826	7.565	H82390	4.865	H39089	4.063	H70778
7.739	T92655	4.998	T54422	9.999	W72342	6.486	W24476
6.577	W01565	6.317	H98046	4.976	T65484	9.999	W01642
9.59	R90884	7.321	N30514	3.745	H96724	7.103	T98068
5.535	R56663	5.137	H38881	9.999	H49809	3.784	AA100388
8.157	W00931	8.737	T99046	3.286	N57334	4.15	H60927
9.999	H50385	5.465	H47440	8.214	R51931	3.897	R28248
8.922	H28503	6.734	N42979	3.912	T79680	7.319	H56754
5.182	H84008	6.802	W01319	3.824	H21568	4.617	R92111
7.947	W01051	7.572	R95136	5.746	T80382	6.422	H94177
9.05	R66879	9.999	AA112231	9.999	R87923	4.375	R87352
9.999	H45773	6.035	H53489	5.902	R23778	6.6	R31364
9.999	AA126109	5.53	N94798	8.584	AA101044	3.713	AA059302
9.999	R54784	6.009	N30964	3.638	AA112340	5.179	H51160
9.999	N98857	9.999	N44142	8.09	R90895	5.423	R25798
9.999	AA056159	7.759	W03129	6.3	N90458	4.448	R63455

ratio	Acc. No.	ratio	Acc. No.	ratio	Acc. No.	ratio	Acc. No.
9.999	R77028	4.156	W03125	4.874	R87886	6.596	T77139
9.999	N36070	7.184	R79618	4.621	N32679	4.909	R63498
5.356	R89245	4.281	H38147	7.755	W02372	4.523	R22272
9.999	H51782	4.445	R38004	4.624	H30637	6.954	H45355
9.999	N48735	9.999	R88190	8.387	R01888	5.624	R75964
5.379	H86672	9.999	N41573	8.644	AA070426	4.742	N32681
9.999	T95210	9.723	H52741	9.999	W31524	4.494	R06552
7.355	R81942	4.401	T54426	6.574	H81786	9.097	R87320
9.999	N53883	4.914	AA054102	3.719	N40437	5.369	R55451
7.685	N24364	6.874	R31243	7.48	N42402	7.075	R84765
7.473	H91761	7.618	AA113044	6.86	R32757	4.058	H84844
4.537	W16945	5.394	R96571	8.926	H21214	6.525	W21173
4.091	DROS-A8	2.424	R71723	4.111	H18154	1.955	AA054211
9.999	AA115819	4.366	H86277	2.981	R53678	3.214	N50075
4.996	N36347	2.214	T53945	3.552	R06539	3.359	T93912
5.587	N30932	5.37	R09668	4.474	H43816	4.156	T77415
2.24	R80470	4.809	H40716	6.663	AA146629	2.779	AA040227
2.746	H16193	2.192	R70132	4.402	R01530	3.468	N26148
6.109	AA004785	2.555	R81899	1.884	H86896	2.744	R26215
3.155	N28396	4.968	N42806	4.013	R68198	3.757	H58331
3.525	AA047581	2.243	H66535	3.632	H16160	2.892	N34217
4.396	H84204	4.049	R46282	1.867	R85333	2.987	AA059324
6.795	R54918	5.522	R49786	1.993	W16980	3.314	R23635
5.972	R78728	8.225	T67978	2.042	T60294	1.29	R77994
4.684	R80601	3.362	R81838	3.009	W32352	3.188	R84692
7.734	R73050	5.364	H19572	1.589	W15194	3.837	N75231
4.832	H12682	4.739	R33409	2.503	H52012	4.043	AA043598
6.87	N56601	3.959	AA069498	2.192	R22397	2.645	R22392
4.153	R80286	4.115	H44733	4.219	R55158	3.933	R11541
9.999	N73428	5.33	AA029010	3.069	AA100975	3.244	H73503
2.754	AA002135	9.999	AA113853	8.61	AA113299	2.909	AA053288
2.948	H39058	4.264	N31362	1.999	R87373	3.059	H38003
6.235	R01799	2.793	R20924	4.285	N45686	4.04	AA088387
6.179	R09942	3.664	R98517	3.87	W02494	4.006	H89896
1.849	R72766	4.656	N24477	3.84	R73063	4.744	AA126881
2.963	R82691	4.443	AA032034	2.614	H14441	6.651	AA088231
2.716	R83247	3.799	AA054203	4.806	AA058898	3.346	N59684
9.375	AA070943	2.852	W95433	1.683	H89823	4.122	T92415
4.051	N43796	5.221	N72527	4.392	AA037418	3.81	AA088190
4.754	N46182	5.408	W02353	4.142	T83106	2.846	N32669
5.892	R32571	4.626	W01717	4.241	T81175	4.096	T58881
3.03	W72046	7.031	W79028	3.335	R31471	2.097	T99924
5.486	H85829	4.702	R12648	3.253	R86304	3.441	AA046822
5.718	R75796	3.427	N32672	2.914	H86156	3.813	H85193
2.697	N45640	3.989	N90836	4.004	H87311	3.134	R91315
2.888	H49806	3.692	N92684	2.595	T94530	3.132	T71293

ratio	Acc. No.	ratio	Acc. No.	ratio	Acc. No.	ratio	Acc. No.
5.419	H52350	2.552	W06829	2.108	H27617	3.45	H79957
4.515	AA071099	2.537	H53375	1.565	H14143	3.198	AA058615
3.535	H51993	4.752	H67462	4.018	H49818	3.309	N34153
3.287	AA069031	4.076	AA029883	1.677	R97857	3.734	H61493
5.108	R79519	4.394	H12075	3.544	N90841	1.687	R56760
5.336	N29042	3.253	T86612	3.901	AA076660	1.796	AA132756
4.075	R48060	9.23	T85558	4.49	N53295	2.934	H20613
4.343	N77703	5.32	N29155	1.67	AA190622	6.358	AA129486
5.491	W56898	3.579	H44664	4.118	AA099647	3.07	H44888
2.202	H28534	5.57	W16814	4.007	AA069114	3.583	W04937
4.449	H62445	2.133	R85191	2.606	W92014	2.521	AA047388
4.196	R90798	2.161	H50471	4.047	W87655	4.349	R52735
7.226	R35606	3.275	H30629	3.891	W01911	2.165	AA065193
3.224	R25899	4.284	R80273	4.32	R54194	4.009	N42453
9.999	AA113119	4.063	H27411	2.085	N57249	3.688	W72149
4.2	N30471	4.231	H44693	3.472	R74281	4.603	AA044942
5.466	T98994	2.471	W47021	4.044	AA054209	3.445	AA187560
2.577	AA075652	4.544	H69415	2.621	H62639	2.273	R74076
2.448	R06926	2.425	AA181061	1.461	T78546	2.268	H65149
2.535	W45582	3.336	T97872	3.677	W40228	5.781	T48877
4.173	T82054	3.553	H51540	6.08	AA101477	3.152	AA033945
3.359	T98110	2.63	N44493	3.907	W00916	3.032	H49111
2.703	R86735	2.587	AA088784	1.846	N90969	3.741	H22503
2.77	AA045397	3.082	R25304	3.006	H83363	2.252	N78086
2.893	R53671	3.06	AA058600	4.484	W17108	2.755	H71635
2.077	AA088407	2.741	H81782	1.482	N64281	2.673	N77126
2.213	T95166	3.466	H84604	2.899	AA045672	4.796	AA083226
1.963	H27400	7.264	AA113169	2.994	H43746		
5.333	T62191	3.859	N63192	2.65	R91004		
2.671	N32657	2.887	N29162	2.439	AA047858		
3.374	T83919	1.926	R28329	2.867	W21593		
3.108	AA191189	3.17	AA172313	2.374	N34202		
2.292	R74157	2.057	R34929	3.144	N80131		
1.787	R21095	4.695	W55902	2.458	AA043774		
4.011	AA180772	2.179	R13273	3.306	T56558		
2.901	H82540	2.354	AA121148	3.896	AA205980		
3.707	R73398	5.805	AA088299	2.565	AA179841		
2.658	H55982	2.233	H84617	2.973	H68783		
1.826	H45128	2.011	AA059207	3.142	W30866		
4.006	W25183	2.742	H39778	2.992	AA028961		
4.299	T56400	3.034	H83022	2.489	R26026		
4.086	T95526	3.245	AA113900	5.751	AA085171		
1.691	AA053901	2.716	R19726	1.822	AA150817		

Example 9. Analysis of Gene Expression under Hypoxia using GEM™ microarrays

The hypoxic induction of genes in FaDu cells was analyzed by comparing the expression of genes in FaDu cells exposed to hypoxic conditions (5% CO_2/5% H_2/90% N_2 for 16 hours at 37°C) to those exposed to normal, oxic conditions. This differential expression was analyzed using GEM™ technology provided by Genome Systems Inc. Messenger RNA (mRNA) was extracted from hypoxic FaDu cells, and separately from oxic FaDu cells.

The total RNA was isolated from the cells essentially according to the standard Genome Systems Inc. protocol, as follows. 500 µl Trizol was added 50-100 mg of fresh frozen cells. The cells were then immediately homogenized. 500 µl Trizol was then added, and the sample was mixed well. The sample was homogenized for five minutes at room temperature. Next, 0.2 ml chloroform was added per 1 ml Trizol. The mixture was shaken vigorously for 15 seconds and then allowed to incubate three minutes at room temperature. The sample was then centrifuged at 12,000X g for 15 minutes at 4°C. The aqueous phase was transferred to a fresh centrifuge tube without disturbing the interphase. 0.5 ml of isopropanol was added and the samples were incubated for 10 minutes at room temperature. The RNA was pelleted by centrifuging at 12,000X g for 10 minutes at 4°C. The supernatant was then removed. 1 ml of 75% ethanol was added to the pellet, which was then vortexed. This was followed by centrifugation at 7,500X g for 5 minutes at 4°C. The ethanol was removed. The pellet was dried for 10 minutes at room temperature and then dissolved in 10 µl nuclease-free water and stored at -80°C.

Next, the poly A+ RNA was isolated from total RNA essentially according to the standard Genome Systems Inc. protocol, as follows. To purify polyA RNA, the total RNA sample was passed twice over OligoTex mRNA isolation columns from Qiagen. After the elution of the polyA RNA, the polyA RNA was ethanol precipitated, and the final product was brought up in DEPC H_2O or TE. For 50 µl of elution from the OligoTex column, 40 µl of 1X TE and 1 µl of glycogen (5 mg/ml) was added. Then

120 µl of 100% EtOH was added and the sample was frozen at -80°C for 10 minutes. The sample was then spun at 12,000 x g for 10 minutes at 4°C. The supernatant was removed and 250 µl of 75% EtOH was added. The pellet was spun at 12,000 x g for 5 minutes at 4°C. The supernatant was again removed and the pellet dried for 10 minutes at room temperature. The pellet was then dissolved in DEPC H2O to a concentration of 50 ng/µl.

The purified RNA samples were sent to Genome Systems Inc. to perform a GEM microarray analysis. From the mRNA samples, fluorescent labeled cDNA probes were prepared by Genome Systems Inc. using standard methodologies familiar to those skilled in the art. The cDNA probes corresponding to the mRNA sample from the oxic FaDu cells were labeled with a different, distinguishable fluorescent label than the cDNA probes corresponding to the mRNA sample from the hypoxic FaDu cells.

The two fluorescent probe samples (one from hypoxic FaDu cells, the other from oxic FaDu cells) were then simultaneously applied by Genome Systems Inc. to their Human UniGEM V microarray for hybridization to the arrayed cDNA molecules. The Human UniGEM V microarray contains sequence verified Genome Systems Inc. proprietary cDNA clones representing more than 4,000 known human genes and up to 3,000 ESTs mapped to the UniGene database. (All of the genes on the microarray were selected for criteria such as known functions, homologies, and presence on the human transcript map.) The genes or gene fragments of the GEM microrarray (each 500-5000 base pairs in length) are arrayed on glass surface to which they have been chemically bonded.

Once the two fluorescent cDNA samples were sufficiently incubated with the arrayed cDNA molecules to allow for hybridization to occur, the microarray was washed free of probe molecules which had not hybridized. The different gene/EST sites of the GEM microarray are then scanned for the each of the two fluorescent labels. Presence of the fluorescent label at a particular gene site indicates the expression of that gene in the cell corresponding to that fluorescent label.

The 30 genes or ESTs which were determined on the microarray to have the greatest level of induction in hypoxic

FaDu cells (versus oxic FaDu cells) are listed below in Table 5, along with their levels of induction, functional category if known, and GenBank accession number.

GEM™ technology was also used to analyze the differential gene expression of Siha cells, C33a cells, and normal keratinocytes as a result of hypoxic induction. The genes or ESTs which were determined on the microarray to have the greatest level of induction in the assayed hypoxic cells (versus oxic cells) including FaDu cells, Siha cells, C33a cells, and normal keratinocytes are shown below in Tables 7, 8, and 9 along with their levels of induction and GenBank accession number. Table 6 illustrates hypoxic gene induction by functional category.

Table 5. Genes Induced by Hypoxia in FaDu cells.

Functional Category	Gene	GenBank #	Induction
Angiogenesis	fibroblast growth factor (FGF-3)	X14445	5.6
	vascular endothelial growth factor (VEGF)	X54936	4.0
	EPH receptor ligand	M57730	3.9
Tissue Remodeling	plasminogen activator inhibitor -1 (PAI-1)	M14083	13.5
	macrophage migration inhibitory factor (MIF)	M25639	9.3
	fibronectin receptor	X06256	6.0
	lysyl hydroxylase-2	U84573	4.8
	endothelin-2	M65199	4.4
Cell Cycle	B-cell translocation gene-1 (BTG-1)	X61123	4.9
	reducing agent and tunicamycin-responsive protein (RTP)	D87953	3.8
	CDC-like kinase (clk-1)	L29219	3.0
	quiescin (Q6)	U97276	2.9
	growth arrest DNA damage-inducible protein 45 (GADD45)	L24498	2.9
Miscellaneous	Differentiation of Embryo Chondrocytes-1 (DEC1)	AB004066	8.2
	low density lipoprotein receptor (LDLR) related protein	X13916	6.0
	human hairy gene homologue (HRY)	L19314	4.7

	adipophilin	X97324	3.9
	cyclooxygenase-1 (COX-1)	U63846	3.0
Metabolism	fructose bisphosphatase	AF054987	4.5
	creatine transporter	U36341, U41163	4.1
	fatty acid binding protein	M94856	3.9
	glucose transporter-like protein III (GLUT-3)	M20681	3.4
	lactate dehyrogenase (LDH)	X02152	2.9
Apoptosis	insulin-like growth factor (IGFBP-3)	M35878	11.1
	Bcl-2-interacting killer (BIK)	X89986	7.6
	19 kDa-interacting protein 3 long/Nip3-like protein X (NipP3L/Nix)	AB004788	5.3
	Pim-1	M54915	4.4
Unknown	EST	AA044768	5.0
	EST	AA054543	4.7
	KIAA0242	D87684	3.9
	EST	AA156240	3.5
	EST	AA186803	3.4
	EST	AA063084	3.0
	EST	H15429	2.9
	EST	U60873	2.9

Example 10. Analysis of Hypoxia-Associated Tumors *In Vivo* by Analyzing Potential Marker Gene Products Present in Blood

Blood samples were collected from patients with solid tumors (head, neck, and cervical malignancies) and the oxygen status of each sample was determined with the use of EF5, a nitroimidazole-binding technique well known in the art (Lord et al., *Cancer Res.* (1993) 53(23):5721-6; and Evans et al., *British Journal of Cancer* (1995) 72(4):875-82).

The blood samples were tested for the presence of plasminogen activator inhibitor-1 (PAI-1). Human serum levels of

PAI-1 protein were determined by using a commercially available ELISA assay called TintElize® kit (Biopool International, Inc.). The assay utilizes a monoclonal antibody that recognizes all forms of human PAI-1 including active, inactive (latent), and complexed to tPA/uPA. The secondary antibody is conjugated to horseradish peroxidase (HRP) and visualization is achieved by conversion of HRP substrate to a yellow-colored product.

According to the TintElize® kit protocol, blood samples are prepared as follows: 9 volumes of blood are collected in 1 volume of 0.1 M trisodium citrate. Alternatively, 99 volumes of blood are collected in 1 volume of 0.5 M EDTA. The samples are then centrifuged at 2500 x g for 15 minutes. During collection, 1/3 of the plasma supernatant is harvested with a plastic pipette. Plasma samples are stored at 2-5°C degrees and assayed within 2 hours. Plasma can be stored for longer periods of time at -20°C and thawed at 37°C for 30 mintues before use. The assay is performed at room temperature according to the TintElize® kit protocol (Biopool International, Inc., Catalog #210221).

PAI-1 levels that were assayed from patient sera were determined to be between 2-20 ng/ml in normal individuals and between 40-110 ng/ml in patients with tumor hypoxia. The increased levels of PAI-1 in the blood stream of patients with tumor hypoxia in comparison to normal individuals establish its use as a diagnostic marker protein.

Various modifications and variations of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art are intended to be within the scope of the claims.

What is claimed is:

1. An array of polynucleotides, comprising at least two different hypoxia-inducible genes, or complements thereto, or at least thirty nucleotide-long fragments thereof, or sequences which hybridize thereto.

2. An array of Claim 1, comprising at least two different polynucleotides, each comprising a hypoxia-inducible gene, or an at least thirty nucleotide-long fragments thereof, or the complement thereto, wherein said hypoxia-inducible genes encode proteins belonging to different functional categories selected from the group consisting of glycolytic enzymes/proteins, metabolic/homeostatic proteins, apoptosis proteins, DNA repair proteins, angiogenesis/tissue remodeling proteins, cell-cycle proteins, erythropoiesis/vascular regulatory proteins, and transcriptional regulatory proteins.

3. An array of Claim 1, comprising at least two different polynucleotides, each comprising a hypoxia-inducible gene, or an at least thirty nucleotide-long fragment thereof, or the complement thereto, wherein said hypoxia-inducible genes all encode proteins belonging to a single functional category selected from the group consisting of glycolytic enzymes/proteins, metabolic/homeostatic proteins, apoptosis proteins, DNA repair proteins, angiogenesis/tissue remodeling proteins, cell-cycle proteins, erythropoiesis/vascular regulatory proteins, and transcriptional regulatory proteins.

4. An array of Claim 1, comprising:

(a) at least one gene selected from the group consisting of *HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage*

migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-1, endothelin-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, insulin-like growth factor binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI11DQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, angiogenin, and SDK3, or an at least thirty nucleotide-long fragment thereof; and

(b) a second polynucleotide, wherein said second polynucleotide comprises a second hypoxia-inducible gene or an at least thirty nucleotide-long fragment thereof.

5. An array of polypeptides, comprising at least two different hypoxia-induced proteins, or biochemically equivalent fragments thereof, wherein each hypoxia-induced protein belongs to a

different functional category selected from the group consisting of glycolytic proteins, metabolic enzymes/proteins, apoptosis proteins, DNA repair proteins, angiogenesis/tissue remodeling proteins, cell-cycle proteins, erythropoiesis/vascular regulatory proteins, and transcriptional regulatory proteins.

6. The array of Claim 5, comprising at least two different hypoxia-induced proteins or biochemically equivalent fragments thereof, wherein said hypoxia-induced proteins are all proteins belonging to a single functional category selected from the group consisting of glycolytic enzymes/proteins, metabolic/homeostatic proteins, apoptosis proteins, DNA repair proteins, angiogenesis/tissue remodeling proteins, cell-cycle proteins, erythropoiesis/vascular regulatory proteins, and transcriptional regulatory proteins.

7. The array of Claim 5, comprising:

(a) at least one protein selected from the group consisting of HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-1, endothelin-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, insulin-like growth factor binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha

5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI1lDQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, angiogenin, and SDK3, or a biochemically equivalent fragment thereof; and

(b) at least one of a second polypeptide, wherein said second polypeptide is a second hypoxia-induced gene product, or a biochemically equivalent fragment thereof.

8. An array of antibodies, comprising:

(a) at least one antibody immunoreactive with a protein selected from the group consisting of HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-1, endothelin-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular endothelial growth factor (VEGF), erythropoietin (EPO),

transferritin, insulin-like growth factor binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI11DQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, angiogenin, and SDK3; and

(b) at least one of a second antibody, wherein said second antibody specifically binds a second hypoxia-induced gene product or a biochemically equivalent fragment thereof.

9. A method of assaying for expression of hypoxia-inducible genes in a tissue of an animal, comprising:

(a) contacting the proteins of a sample of body fluid or tissue obtained from said animal with the array of Claim 7; and

(b) detecting the amount and position of protein from said sample that binds to the array.

10. A method of evaluating a hypoxia-related condition in a tissue of an animal, comprising:

(a) contacting the proteins of a sample of body fluid or tissue obtained from said animal with the array of Claim 7; and

(b) detecting the amount and position of protein from said sample that binds to the array.

11. A method of determining the presence of hypoxia in a tissue in an animal, comprising:

(a) contacting the proteins of a sample of body fluid or tissue obtained from said animal with the array of Claim 7; and

(b) detecting the amount and position of protein from said sample that binds to the array.

12. A method of assaying for expression of hypoxia-inducible genes in a tissue of an animal, comprising:

(a) contacting messenger RNA from a sample of body fluid or tissue obtained from said animal, or cDNA derived therefrom, with the array of Claim 1; and

(b) detecting the amount and position of messenger RNA or cDNA from said sample that binds to the array.

13. A method of evaluating a hypoxia-related condition in a tissue of an animal, comprising:

(a) contacting messenger RNA from a sample of body fluid or tissue obtained from said animal, or cDNA derived therefrom, with the array of Claim 1; and

(b) detecting the amount and position of the messenger RNA or the cDNA that binds to the array.

14. A method of determining the presence of hypoxia in a tissue in an animal, comprising:

(a) contacting messenger RNA from a sample of body fluid or tissue obtained from said animal, or cDNA derived therefrom, with the array of Claim 1; and

(b) detecting the amount and position of the messenger RNA or the cDNA that binds to the array.

15. A method of determining the presence of hypoxia in a tissue in an animal, comprising:

assaying for either the mRNA transcript or the polypeptide expression product of a gene selected from the group consisting of *HIG1, HIG2, annexin V, lipocortin 2, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), Ku autoantigen, phosphoribosylpyrophosphate synthetase, acetoacetylCoA thiolase, ribosomal L7, fibroblast growth factor-3 (FGF-3), EPH receptor ligand, plasminogen activator inhibitor-1 (PAI-1), macrophage migration inhibitory factor (MIF), fibronectin receptor, fibronectin 1, lysl hydroxylase, lysyl hydroxylase-2, endothelin-*

1, endothelin-2, B-cell translocation gene-1 (BTG-1), reducing agent and tunicamycin-responsive protein (RTP), CDC-like kinase-1 (clk-1), quiescin, growth arrest DNA damage-inducible protein 45 (GADD45), DNA damage-inducible transcript I24498, differentiation of embryo chondrocytes (DEC1), low density lipoprotein receptor related protein (LDLR), hamster hairy gene homologue, adipophilin, cyclooxygenase-1 (COX-1), fructose bisphosphatase, creatine transporter, fatty acid binding protein, lactate dehydrogenase (LDH), Bcl-2-interacting killer (BIK), 19 kDa-interacting protein 3, Nip3L/Nix, Pim-1, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, insulin-like growth factor binding protein 3 (IGFBP-3), phosphofructokinase (PFK), aldolase A, aldolase C, integrin alpha 5, integrin alpha 5 receptor, placental growth factor, interleukin-1 (IL-1) receptor, APO-1 (Fas receptor), LDHM, phosphoglycerate kinase 1 (PGK-1), monocarboxylate transporter 3, DNA binding protein A20, peroxisome proliferation receptor, trisephosphate isomerase, Ig associated alpha, interferon regulatory factor 6 (IRF6), putative ORF KIAA0113, c-fos, glucose transporter-like protein 3/glucose transporter isoform 3 (GLUT-3), glycogen branching enzyme, TGF beta, brain HHCPA78, mucin 1, RNAse L, Mxi 1, glucose-regulated protein 78 (GRP78), quiescin, lysl oxidase, prostaglandin endoperoxide synthetase, insulin-inducible protein 1, MHC-cI1lDQB, myocyte-specific factor 2 (MEF2), bacteria permeating protein, hexokinase, Cap43 (nickel inducible), cyclin G2, carbonic anhydrase IX, TPI, angiogenin, and SDK3 in a body fluid or the tissue of said animal.

16. The method of Claim 15, wherein said tissue is a tumor.

17. An array of polynucleotides, comprising:

(a) at least one hypoxia-inducible gene, or the complement thereto, or an at least thirty nucleotide-long fragment thereof, or a sequence which hybridizes thereto, wherein said hypoxia-inducible gene is bound to a solid surface; and

(b) at least one hypoxia-repressible gene, or the complement thereto, or an at least thirty nucleotide-long

fragment thereof, or a sequence which hybridizes thereto, wherein said hypoxia-repressible gene is bound to said solid surface.

18. A method of assaying for differential expression of hypoxia-related genes in a tissue of an animal, comprising:

(a) contacting messenger RNA from a sample of body fluid or tissue obtained from said animal, or cDNA derived therefrom, with the array of Claim 17; and

(b) detecting the amount and position of the messenger RNA or the cDNA that binds to the array.

19. An array of polynucleotides, comprising at least one hypoxia-repressible gene, or the complement thereto, or an at least thirty nucleotide-long fragment thereof, or a sequence which hybridizes thereto, wherein said hypoxia-repressible gene is bound to a solid surface.

20. A method of assaying for differential expression of hypoxia-repressible genes in a tissue of an animal, comprising:

(a) contacting messenger RNA from a sample of body fluid or tissue obtained from said animal, or cDNA derived therefrom, with the array of Claim 19; and

(b) detecting the amount and position of the messenger RNA or the cDNA that binds to the array.

21. A method for detecting the presence of tumor hypoxia in body fluid, comprising:

(a) measuring the amount of a marker protein in said body fluid; and

(b) quantifying the amount of said marker protein, wherein said marker protein is selected from the group consisting of PAI-1, IGF-BP3, placental growth factor, adipophilin, mucin 1, endothelin-1, endothelin-2, vascular endothelial growth factor (VEGF), erythropoietin (EPO), transferritin, EPH receptor ligand, angiogenin, and TGF beta.

22. A method for detecting the presence of a hypoxic condition in a tissue or body fluid of an animal, which method

comprises assaying a sample of said tissue or body fluid for the presence of the expression products of one or more genes of claim 4.

23. A method for detecting the presence of a hypoxic condition in a tissue or body fluid of an animal, which method comprises assaying a sample of said tissue or body fluid for the presence of one or more polypeptides of claim 7.

24. A method for detecting the presence of a hypoxic condition in a tissue or body fluid of an animal, which method comprises assaying a sample of said tissue or body fluid for the presence of one or more antibodies of claim 8.

FIG 1A

CGGAAGCCGG TTGGGGTGTG AGAGGTTTTC TCGCTCTAGG GAGATTCTTC
AAGCAATCAC TATGTCAACA GACACAGGTG TTTCCCTTCC TTCATATGAG
GAAGATCAGG GATCAAAACT CATTCGAAAA GCTAAAGAGG CACCATTCGT
ACCCGTTGGA ATAGCGGGTT TTGCAGCAAT TGTTGCATAT GGATTATATA
AACTGAAGAG CAGGGGAAAT ACTAAAATGT CCATTCATCT GATCCACATG
CGTGTGGCAG CCCAAGGCTT TGTTGTAGGA GCAATGACTG TTGGTATGGG
CTATTCCATG TATCGGGAAT TCTGGGCAAA ACCTAAGCCT TAGAAGAAGA
GATGCTGTCT TGGTCTTGTT GGAGGAGCTT GCTTTAGTTA GATGTCTTAT
TATTAAAGTT ACCTATTATT GTTGGAAATA AACTAATTTG TATGGGTTTA
GATGGTAACA TGGCATTTTG AATATTGGCT TCCTTTCTTG CAGGCTTGAT
TTGCTTGGTG ACCGAATTAC TAGTGACTAG TTTACTAACT AGGTCATTCA
AGGAAGTCAA GTTAACTTAA ACATGTCACC TAAATGCACT TGATGGTGTT
GAAATGTCCA CCTTCTTAAA TTTTTAAGAT GAACTTAGTT CTAAAGAAGA
TAACAGGCCA ATCCTGAAGG TACTCCCTGT TTGCTGCAGA ATGTCAGATA
TTTTGGATGT TGCATAAGAG TCCTATTTGC CCCAGTTAAT TCAACTTTTG
TCTGCCTGTT TTGTGGACTG GCTGGCTCTG TTAGAACTCT GTCCAAAAAG
TGCATGGAAT ATAACTTGTA AAGCTTCCCA CAATTGACAA TATATATGCA
TGTGTTTAAA CCAAATCCAG AAAGCTTAAA CAATAGAGCT GCATAATAGT
ATTTATTAAA GAATCACAAC TGTAAACATG AGAATAACTT AAGGATTCTA
GTTTAGTTTT TTGTAATTGC AAATTATATT TTTGCTGCTG ATATATTAGA
ATAATTTTTA AATGTCATCT TGAAATAGAA ATATGTATTT TAAGCACTCA
CGCAAAGGTA AATGAACACG TTTTAAATGT GTGTGTTGCT AATTTTTTCC
ATAAGAATTG TAAACATTGA ACTGAACAAA TTACCTATAA TGGATTTGGT
TAATGACTTA TGAGCAAGCT GGTTTGGCCA GACAGTATAC CCAAACTTTT
ATATAATATA CAGAAGGCTA TCACACTTGT GAAATTCTCT TGTCTAATCT
GAATTTGCAT TCCATGGTGT TAACATGGTA TATGTATTGT TATTAAAGTA
AGTGACCCAT GTC

FIG 1B

MSTDTGVSLP SYEEDQGSKL IRKAKEAPFV PVGIAGFAAI VAYGLYKLKS
RGNTKMSIHL IHMRVAAQGF VVGAMTVGMG YSMYREFWAK PKP

FIG 2A

ACAAAACTGG AGTCCACCGC GGTGGCGGCC GCTCTAGAAT AGTGGATCCC
CCGGGCTGCA GGAATTCGGC ACGAGGGCGC TTTTGTCTCC GGTGAGTTTT
GTGGCGGGAA GCTTCTGCGC TGGTGCTTAG TAACCGACTT TCCTCCGGAC
TCCTGCACGA CCTGCTCCTA CAGCCGGCGA TCCACTCCCG GCTGTTCCCC
CGGAGGGTCC AGAGGCCTTT CAGAAGGAGA AGGCAGCTCT GTTTCTCTGC
AGAGGAGTAG GGTCCTTTCA GCCATGAAGC ATGTGTTGAA CCTCTACCTG
TTAGGTGTGG TACTGACCCT ACTCTCCATC TTCGTTAGAG TGATGGAGTC
CCTAGAAGGC TTACTAGAGA GCCCATCGCC TGGGACCTCC TGGACCACCA
GAAGCCAACT AGCCAACACA GAGCCCACCA AGGGCCTTCC AGACCATCCA
TCCAGAAGCA TGTGATAAGA CCTCCTTCCA TACTGGCCAT ATTTTGGAAC
ACTGACCTAG ACATGTCCAG ATGGGAGTCC CATTCCTAGC AGACAAGCTG
AGCACCGTTG TAACCAGAGA ACTATTACTA GGCCTTGAAG AACCTGTCTA
ACTGGATGCT CATTGCCTGG GCAAGGCCTG TTTAGGCCGG TTGCGGTGGC
TCATGCCTGT AATCCTAGCA CTTTGGGAGG CTGAGGTGGG TGGATCACCT
GAGGTCAGGA GTTCGAGACC AGCCTCGCCA ACATGGCGAA ACCCCATCTC
TACTAAAAAT ACAAAAGTTA GCTGGGTGTG GTGGCAGAGG CCTGTAATCC
CAGTTCCTTG GGAGGCTGAG GCGGGAGAAT TGCTTGAACC CGGGGACGGA
GGTTGCAGTG AACCGAGATC GCACTGCTGT ACCCAGCCTG GGCCACAGTG
CAAGACTCCA TCTCAAAAAA AAAAAGAAAA GAAAAAGCCT GTTTAATGCA
CAGGTGTGAG TGGATTGCTT ATGGCTATGA GATAGGTTGA TCTCGCCCTT
ACCCCGGGGT CTGGTGTATG CTGTGCTTTC CTCAGCAGTA TGGCTCTGAC
ATCTCTTAGA TGTCCCAACT TCAGCTGTTG GGAGATGGTG ATATTTTCAA
CCCTACTTCC TAAACATCTG TCTGGGGTTC CTTTAGTCTT GAATGTCTTA
TGCTCAATTA TTTGGTGTTG AGCCTCTCTT CCACAAGAGC TCCTCCATGT
TTGGATAGCA GTTGAAGAGG TTGTGTGGGT GGGCTGTTGG GAGTGAGGAT
GGAGTGTTCA GTGCCCATTT CTCATTTTAC ATTTTAAAGT CGTTCCTCCA
ACATAGTGTG TATTGGTCTG AAGGGGGTGG TGGGATGCCA AAGCCTGCTC
AAGTTATGGA CATTGTGGCC ACCATGTGGC TTAAATGATT TTTTCTAACT
AATAAAGTGG AATATATATT TCAAAAAAAA AAAAAAAAAA CTCGAGGGGG
GCCCGTACCC AATCGC

FIG 2B

MKHVLNLYLL GVVLTLLSIF VRVMESLEGL LESPSPGTSW TTRSQLANTE
PTKGLPDHPS RSM

FIG 3A

GGCCAGAAAC CGGCAGACTC GGAAGGGACC CCGCGTCTCG GAAGACTCTT
CAAGAAATCA CAATGTCAAC CAACACAGAC CTTTCTCTCT CTTCATACGA
TGAAGGTCAG GGGTCTAAGT TTATTCGGAA AGCTAAGGAG ACACCGTTTG
TCCCCATTGG AATGGCGGGC TTTGCAGCGA TTGTTGCCTA TGGGTTGTAC
AAGCTGAAGA GCAGAGGAAA TACAAAGATG TCCATTCACT TGATCCACAT
GCGTGTAGCA GCCCAGGGCT TTGTTGTGGG GGCCATGACT CTTGGTATGG
GCTACTCCAT GTATCAGGAA TTCTGGGCCA ACCCTAAGCC TAAGCCTTAG
AAGAGCTGGT GGCATGGGAA GTGCTTGCTT TAGTTAGACG TCTCATATTG
AGGTTACGTG TTTGTATCTA CAATAAATAA CATGTGGGTT TAGA

FIG 3B

MSTNTDLSLS SYDEGQGSKF IRKAKETPFV PIGMAGFAAI VAYGLYKLKS
RGNTKMSIHL IHMRVAAQGF VVGAMTLGMG YSMYQEFWAN PKPKP

FIG 4A

CGTCAGGCAA AATTACTTCC TCCAGACTGT ACGAGGGATC TGTGGCTCCA
AAGACTCATA AAATAATAAT AATTCTTTAC AGACGATTCA AGAGACACTT
CTTAAACAGT CAGGATGACT GCCTATGATG AGAATGAATC CAAGTTAATG
CGAAAAGTAA AGGAGAATCC ATTTGTCCCA GTGGGGATTG CTGGATTCTT
TGCCATTGTT GGGTACAGAC TGATGAAAAT GAAAAATCGG GGAGACACAA
AAATGTCGGT ACACCTGATC CACATGCGTG TAGCTGCACA AGGCTTTGTG
GTCGGAGCCA TGACTGTTGG AGTCCTGTAT TCAATGTACA GAGATTTCAT
TGTAAAACCC AGAGAAGAAC AGAAATCAAT GCAAAACAAG TGAACACCAC
CTCTTACCCT GGTATTTTAT GTCCCTTAAT ATTACCTCAT ATTAAGGTGT
GTAGAGTGTT TATTTTTACT GATGGGTCAA CTTTTATATG CACGCATCAC
TCTAGAAGCT CCCCTCTACT GTAAATACCC GTAACTTATT GATCACACTT
GACATCTTCT AAGTATCATA CCAGAGGGTC AAGTTGTCAC TTCTGTATTG
AGAAGGAGTT ATATTGATCT CAGCTGTTTT AACACTGGTT ACACTCCTTG
TGTTTGCGGT TATAAACGTA GCTGGTTTGA TATCTGTGTA GACAGATGAC
ATTACTGAGT GCAGAGTTTT TAGAGCCTCT TATTGTGATG TAGTGTGTGT
GAAATGGCGA GAAGCCTCGA ATTTACGGCA CACGTATCAA TTGTAAACAC
GTATGTCCAT GGCAAAGTCT GGATTTTAAG ACACCCCACC AATTGGCAGG
TTGCCCAACA GGTTTCTTCC TCCGGCCGGA ATTTAATGTC TCGTACCAGC
TATATTGTTT TATGTACTAA TTTAGGAACT TTTTGCCAAA TAAAAAAATG
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TCTTGAGGAA GGCTTTGACA ACACTTGACT AACAGACGAA ACCTAAGCTC
CCACATTGTT TAAACAACTA GAACACAAGA GGTTTTTGAC TCACAACAGC
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GAAAAAAGGA CTATGCATAT TTCTTGACCG AAACTATAAA GATCTCTTGT
AGAATTAAAA TGGATTATTT TAATTTGGTA CGCTTTCCAC AAAATGTCTT
TTTTTTTTTT TGACACAAGG GGGTCCAATA TTTCAATAGA GCAGCTTCAC
AAGCCCTCAC GAGAAATGTA AACCAACTGA CACCTTCACC TGTACAGACT
GTGCAGTAAT CTATAGTATA TCATCATATA GCCTACCTTG TGATAAGCTT
AAATAGATGC CTTGTTAAGT TATACACAAA GTTGAATTTT GAATATTGTG
TGCAAATACA GAAGATGTTA TTGAATGTTT TTTTTTCCTC TCGAAATAAA
ATTGACCAGT CTTGTAATTC T

FIG 4B

MTAYDENESK LMRKVKENPF VPVGIAGFFA IVGYRLMKMK NRGDTKMSVH
LIHMRVAAQG FVVGAMTVGV LYSMYRDFIV KPREEQKSMQ NK

FIG 5A

TCGGACGAGG GCCTCGCAGA AGGGCGGGCT TTGGGAGGTC CGTTTGTCTT
TGGGGCTTAT TTCTATCCAG AGCAGTGCCT GCGTGGAGCT TCCACGTTGC
GACTCAGCCG ACCTTCTTCC TTACTCCTGC ACGACCTGGT GTGACTGTGA
GCAGCCGTCT CTCAACTTTT CCTTCTGAGG ATCTAGCAGC AGAAAGCAGC
TCTACTTCCC TGCAAAGGAG CTGGGCACCG TCGCCATGAA GTTCATGCTG
AACCTCTATG TGCTGGGCAT CATGTTGACC CTGCTTTCCA TCTTTGTTAG
AGTGATGGAG TCTCTGGGAG GCTTACTGGA GAGCCCACTG CCCGGGAGCT
CCTGGATCAC GAGGGGTCAG CTAGCCAACA CACAGCCTCC TAAGGGCCTG
CCAGACCATC CATCCCGAGG AGTGCAGTGA ACCTCCCTCC CTGCAGGCAT
CACAGCTTCA GCATGTCCAA CCACACGTTC CATTTCTCGG GAGGCAGCAT
CAAGTGTCTC CAAAGGACTC TTACTAGGCC TGGAAGGGCT GTTCCCTTAC
CCTGGAAAAG AGCCTATTTC CCCTAGAGCT GTGAGTGGGC TGTCTGTGGC
TCTGGGATGG AGGTGTACCA GTTCCAGCTG TAGGGAGAAT GGATTTTGGT
TTCGTTTGTT TCAGACCTCT GTCCTAAAGG ACTCTTTTGG ACCTAAGTAT
CTTCTGTTGG TTTACCATTG AGTCTTTCC CTGAGAGTTG TTTGGATGGC
ATCAAAGGGG TTGTGGTTTG ACTGTGAAGA CAGAGGGTGG ACTATCCAGT
GTCCAGGTCA AGTTGTACAT TTAAGTTCTT TCTCCAGTGT AATGCACATG
TGTTTGT

FIG 5B

MKFMLNLYVL GIMLTLLSIF VRVMESLGGL LESPLPGSSW ITRGQLANTQ
PPKGLPDHPS RGVQ

FIG 6A

```
hHIG1        1  MSTDTGVSLPSYEEDQGSKLIRKAKEAPFVPVGIAGFAAIVAYGLYKLKS
mHIG1        1  MSTNTDLSLSSYDEGQGSKFIRKAKETPFVPIGMAGFAAIVAYGLYKLKS
GHL1(fish)   1         MTAYDENE-SKLMRKVKENPFVPVGIAGFFAIVGYRLMKMKN

hHIG1       51  RGNTKMSIHLIHMRVAAQGFVVGAMTVGMGYSMYREFWAKPKP
mHIG1       51  RGNTKMSIHLIHMRVAAQGFVVGAMTLGMGYSMYQEFWANPKPKP
GHL1(fish)  42  RGDTKMSVHLIHMRVAAQGFVVGAMTVGVLYSMYRDFIVKPREEQKSMQNK
```

FIG 6B

```
hHIG2        1  MKHVLNLYLLGVVLTLLSIFVRVMESLEGLLESPSPGTSWTTRSQLANTE
mHIG2        1  MKFMLNLYVLGIMLTLLSIFVRVMESLGGLLESPLPGSSWITRGQLANTQ

hHIG2       51  PTKGLPDHPSRSM
mHIG2       51  PPKGLPDHPSRGVQ
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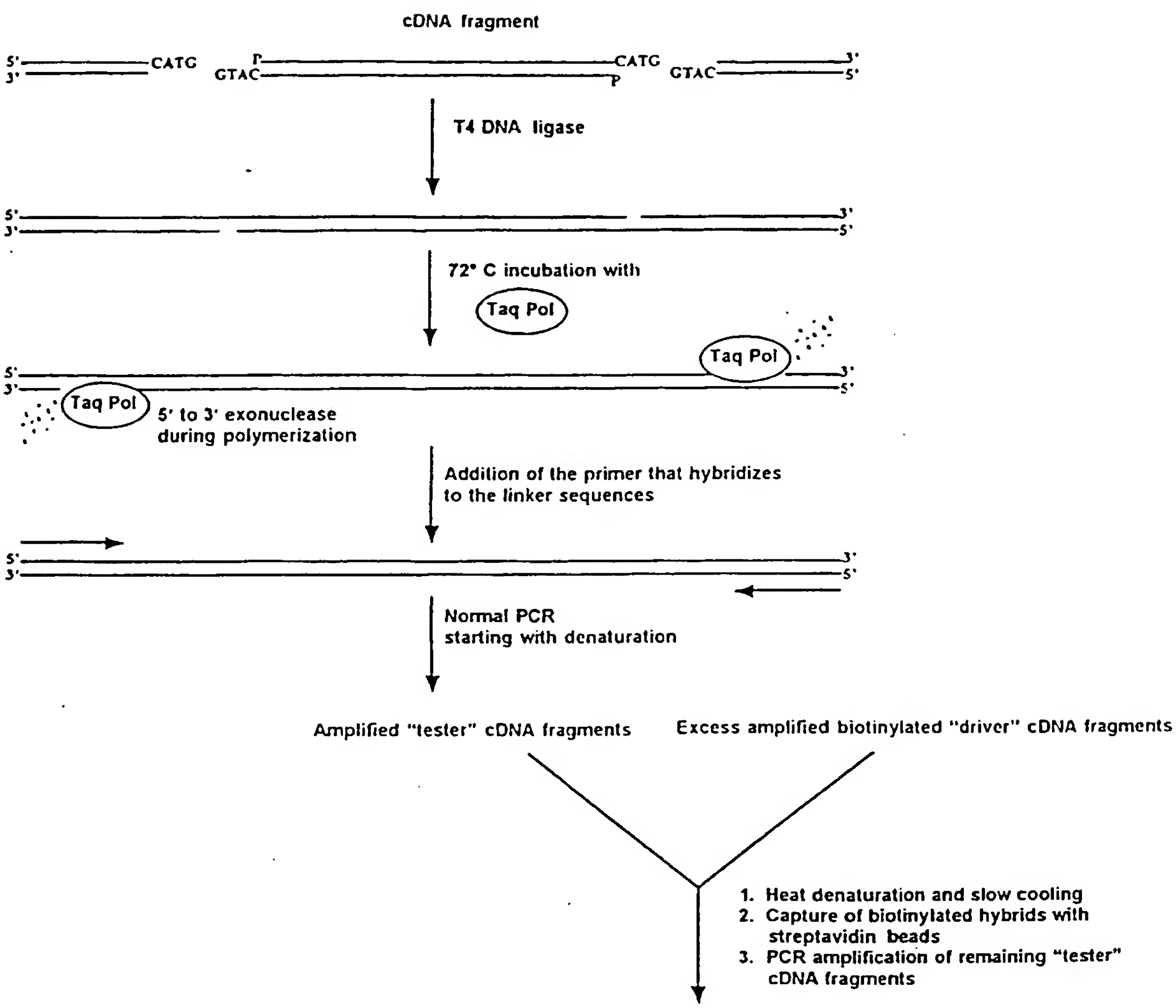
cDNA fragment
5' CATG
3'
GTAC
CATG
GTAC
3'
5'
T4 DNA ligase
72° C incubation with
Taq Pol
Taq Pol
Taq Pol
5' to 3' exonuclease
during polymerization
Addition of the primer that hybridizes
to the linker sequences
Normal PCR
starting with denaturation
Amplified "tester" cDNA fragments
Excess amplified biotinylated "driver" cDNA fragments
1. Heat denaturation and slow cooling
2. Capture of biotinylated hybrids with
streptavidin beads
3. PCR amplification of remaining "tester"
cDNA fragments

FIG 7

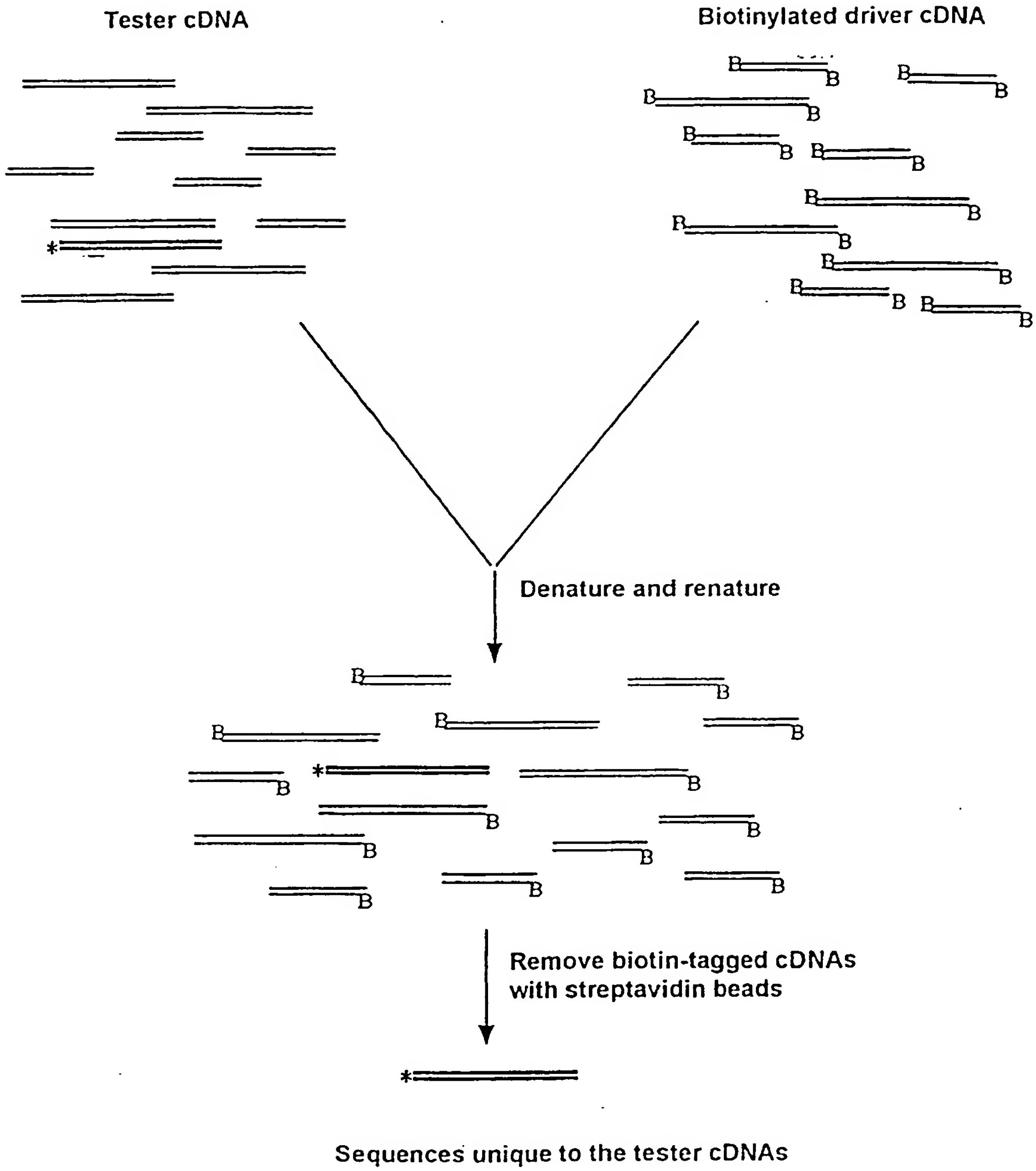
Tester cDNA
Biotinylated driver cDNA
Denature and renature
Remove biotin-tagged cDNAs
with streptavidin beads
Sequences unique to the tester cDNAs

FIG 8

SEQUENCE LISTING

<110> Denko, Nicholas C.
Giaccia, Amato J.
Green, Christopher J.
Laderoute, Keith R.
Schindler, Cornelia
Koong, Albert C.

<120> Hypoxia-Related Human Genes, Proteins, and Uses Thereof

<130> 15907-0011P2

<140> US 09/410,375
<141> 1999-09-30

<150> US 09/280,190
<151> 1999-03-29

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  Met Ser Thr Asp Thr Gly Val Ser Leu Pro Ser Tyr Glu Glu Asp Gln
    1               5                  10                  15

gga tca aaa ctc att cga aaa gct aaa gag gca cca ttc gta ccc gtt   157
Gly Ser Lys Leu Ile Arg Lys Ala Lys Glu Ala Pro Phe Val Pro Val
            20                  25                  30

gga ata gcg ggt ttt gca gca att gtt gca tat gga tta tat aaa ctg   205
Gly Ile Ala Gly Phe Ala Ala Ile Val Ala Tyr Gly Leu Tyr Lys Leu
```

```
        35                  40                  45

aag agc agg gga aat act aaa atg tcc att cat ctg atc cac atg cgt    253
Lys Ser Arg Gly Asn Thr Lys Met Ser Ile His Leu Ile His Met Arg
    50                  55                  60

gtg gca gcc caa ggc ttt gtt gta gga gca atg act gtt ggt atg ggc    301
Val Ala Ala Gln Gly Phe Val Val Gly Ala Met Thr Val Gly Met Gly
65                  70                  75                  80

tat tcc atg tat cgg gaa ttc tgg gca aaa cct aag cct tagaagaaga     350
Tyr Ser Met Tyr Arg Glu Phe Trp Ala Lys Pro Lys Pro
                85                  90

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acctattatt gttggaaata aactaatttg tatgggttta gatggtaaca tggcattttg 470

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taacaggcca atcctgaagg tactccctgt ttgctgcaga atgtcagata ttttggatgt 710

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gctggctctg ttagaactct gtccaaaaag tgcatggaat ataacttgta aagcttccca 830

caattgacaa tatatatgca tgtgtttaaa ccaaatccag aaagcttaaa caatagagct 890

gcataatagt atttattaaa gaatcacaac tgtaaacatg agaataactt aaggattcta 950

gtttagtttt ttgtaattgc aaattatatt tttgctgctg atatattaga ataattttta 1010

aatgtcatct tgaaatagaa atatgtattt taagcactca cgcaaaggta aatgaacacg 1070

ttttaaatgt gtgtgttgct aattttttcc ataagaattg taaacattga actgaacaaa 1130

ttacctataa tggatttggt taatgactta tgagcaagct ggtttggcca gacagtatac 1190

ccaaactttt atataatata cagaaggcta tcacacttgt gaaattctct tgtctaatct 1250

gaatttgcat tccatggtgt taacatggta tatgtattgt tattaaagta agtgacccat 1310

gtc                                                               1313
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<210> 2
<211> 93
<212> PRT
<213> Homo sapiens

<400> 2
Met Ser Thr Asp Thr Gly Val Ser Leu Pro Ser Tyr Glu Glu Asp Gln
1   5   10   15

Gly Ser Lys Leu Ile Arg Lys Ala Lys Glu Ala Pro Phe Val Pro Val
            20   25   30

Gly Ile Ala Gly Phe Ala Ala Ile Val Ala Tyr Gly Leu Tyr Lys Leu
        35   40   45

Lys Ser Arg Gly Asn Thr Lys Met Ser Ile His Leu Ile His Met Arg
    50   55   60

Val Ala Ala Gln Gly Phe Val Val Gly Ala Met Thr Val Gly Met Gly
65   70   75   80

Tyr Ser Met Tyr Arg Glu Phe Trp Ala Lys Pro Lys Pro
                85   90

<210> 3
<211> 1466
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (274)..(462)

<400> 3
acaaaactgg agtccaccgc ggtggcggcc gctctagaat agtggatccc ccgggctgca 60

ggaattcggc acgagggcgc ttttgtctcc ggtgagtttt gtggcgggaa gcttctgcgc 120

tggtgcttag taaccgactt tcctccggac tcctgcacga cctgctccta cagccggcga 180

tccactcccg gctgttcccc cggagggtcc agaggccttt cagaaggaga aggcagctct 240

gtttctctgc agaggagtag ggtcctttca gcc atg aag cat gtg ttg aac ctc 294
Met Lys His Val Leu Asn Leu
1   5

```
tac ctg tta ggt gtg gta ctg acc cta ctc tcc atc ttc gtt aga gtg   342
Tyr Leu Leu Gly Val Val Leu Thr Leu Leu Ser Ile Phe Val Arg Val
        10                  15                  20

atg gag tcc cta gaa ggc tta cta gag agc cca tcg cct ggg acc tcc   390
Met Glu Ser Leu Glu Gly Leu Leu Glu Ser Pro Ser Pro Gly Thr Ser
    25                  30                  35

tgg acc acc aga agc caa cta gcc aac aca gag ccc acc aag ggc ctt   438
Trp Thr Thr Arg Ser Gln Leu Ala Asn Thr Glu Pro Thr Lys Gly Leu
 40                  45                  50                  55

cca gac cat cca tcc aga agc atg tgataagacc tccttccata ctggccatat  492
Pro Asp His Pro Ser Arg Ser Met
                 60

tttggaacac tgacctagac atgtccagat gggagtccca ttcctagcag acaagctgag 552

caccgttgta accagagaac tattactagg ccttgaagaa cctgtctaac tggatgctca 612

ttgcctgggc aaggcctgtt taggccggtt gcggtggctc atgcctgtaa tcctagcact 672

ttgggaggct gaggtgggtg gatcacctga ggtcaggagt tcgagaccag cctcgccaac 732

atggcgaaac cccatctcta ctaaaaatac aaaagttagc tgggtgtggt ggcagaggcc 792

tgtaatccca gttccttggg aggctgaggc gggagaattg cttgaacccg gggacggagg 852

ttgcagtgaa ccgagatcgc actgctgtac ccagcctggg ccacagtgca agactccatc 912

tcaaaaaaaa aaagaaaaga aaaagcctgt ttaatgcaca ggtgtgagtg gattgcttat 972

ggctatgaga taggttgatc tcgcccttac cccggggtct ggtgtatgct gtgctttcct 1032

cagcagtatg gctctgacat ctcttagatg tcccaacttc agctgttggg agatggtgat 1092

attttcaacc ctacttccta aacatctgtc tggggttcct ttagtcttga atgtcttatg 1152

ctcaattatt tggtgttgag cctctcttcc acaagagctc ctccatgttt ggatagcagt 1212

tgaagaggtt gtgtgggtgg gctgttggga gtgaggatgg agtgttcagt gcccatttct 1272

cattttacat tttaaagtcg ttcctccaac atagtgtgta ttggtctgaa gggggtggtg 1332

ggatgccaaa gcctgctcaa gttatggaca ttgtggccac catgtggctt aaatgatttt 1392

ttctaactaa taaagtggaa tatatatttc aaaaaaaaaa aaaaaaaact cgaggggggc 1452
```

ccgtacccaa tcgc 1466

<210> 4
<211> 63
<212> PRT
<213> Homo sapiens

<400> 4
Met Lys His Val Leu Asn Leu Tyr Leu Leu Gly Val Val Leu Thr Leu
1 5 10 15

Leu Ser Ile Phe Val Arg Val Met Glu Ser Leu Glu Gly Leu Leu Glu
20 25 30

Ser Pro Ser Pro Gly Thr Ser Trp Thr Thr Arg Ser Gln Leu Ala Asn
35 40 45

Thr Glu Pro Thr Lys Gly Leu Pro Asp His Pro Ser Arg Ser Met
50 55 60

<210> 5
<211> 444
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (63)..(347)

<400> 5
ggccagaaac cggcagactc ggaagggacc ccgcgtctcg gaagactctt caagaaatca 60

ca atg tca acc aac aca gac ctt tct ctc tct tca tac gat gaa ggt 107
Met Ser Thr Asn Thr Asp Leu Ser Leu Ser Ser Tyr Asp Glu Gly
1 5 10 15

cag ggg tct aag ttt att cgg aaa gct aag gag aca ccg ttt gtc ccc 155
Gln Gly Ser Lys Phe Ile Arg Lys Ala Lys Glu Thr Pro Phe Val Pro
20 25 30

att gga atg gcg ggc ttt gca gcg att gtt gcc tat ggg ttg tac aag 203
Ile Gly Met Ala Gly Phe Ala Ala Ile Val Ala Tyr Gly Leu Tyr Lys
35 40 45

```
ctg aag agc aga gga aat aca aag atg tcc att cac ttg atc cac atg    251
Leu Lys Ser Arg Gly Asn Thr Lys Met Ser Ile His Leu Ile His Met
        50                  55                  60

cgt gta gca gcc cag ggc ttt gtt gtg ggg gcc atg act ctt ggt atg    299
Arg Val Ala Ala Gln Gly Phe Val Val Gly Ala Met Thr Leu Gly Met
    65                  70                  75

ggc tac tcc atg tat cag gaa ttc tgg gcc aac cct aag cct aag cct    347
Gly Tyr Ser Met Tyr Gln Glu Phe Trp Ala Asn Pro Lys Pro Lys Pro
 80                  85                  90                  95

tagaagagct ggtggcatgg gaagtgcttg ctttagttag acgtctcata ttgaggttac 407

gtgtttgtat ctacaataaa taacatgtgg gtttaga                          444


<210> 6
<211> 95
<212> PRT
<213> Mus musculus

<400> 6
Met Ser Thr Asn Thr Asp Leu Ser Leu Ser Ser Tyr Asp Glu Gly Gln
  1               5                  10                  15

Gly Ser Lys Phe Ile Arg Lys Ala Lys Glu Thr Pro Phe Val Pro Ile
             20                  25                  30

Gly Met Ala Gly Phe Ala Ala Ile Val Ala Tyr Gly Leu Tyr Lys Leu
         35                  40                  45

Lys Ser Arg Gly Asn Thr Lys Met Ser Ile His Leu Ile His Met Arg
     50                  55                  60

Val Ala Ala Gln Gly Phe Val Val Gly Ala Met Thr Leu Gly Met Gly
 65                  70                  75                  80

Tyr Ser Met Tyr Gln Glu Phe Trp Ala Asn Pro Lys Pro Lys Pro
                 85                  90                  95


<210> 7
<211> 1521
<212> DNA
<213> Seriola quinqueradiata
```

<220>
<221> CDS
<222> (115)..(390)

<400> 7
cgtcaggcaa aattacttcc tccagactgt acgagggatc tgtggctcca aagactcata 60

aaataataat aattctttac agacgattca agagacactt cttaaacagt cagg atg 117
Met
1

act gcc tat gat gag aat gaa tcc aag tta atg cga aaa gta aag gag 165
Thr Ala Tyr Asp Glu Asn Glu Ser Lys Leu Met Arg Lys Val Lys Glu
5 10 15

aat cca ttt gtc cca gtg ggg att gct gga ttc ttt gcc att gtt ggg 213
Asn Pro Phe Val Pro Val Gly Ile Ala Gly Phe Phe Ala Ile Val Gly
20 25 30

tac aga ctg atg aaa atg aaa aat cgg gga gac aca aaa atg tcg gta 261
Tyr Arg Leu Met Lys Met Lys Asn Arg Gly Asp Thr Lys Met Ser Val
35 40 45

cac ctg atc cac atg cgt gta gct gca caa ggc ttt gtg gtc gga gcc 309
His Leu Ile His Met Arg Val Ala Ala Gln Gly Phe Val Val Gly Ala
50 55 60 65

atg act gtt gga gtc ctg tat tca atg tac aga gat ttc att gta aaa 357
Met Thr Val Gly Val Leu Tyr Ser Met Tyr Arg Asp Phe Ile Val Lys
70 75 80

ccc aga gaa gaa cag aaa tca atg caa aac aag tgaacaccac ctcttaccct 410
Pro Arg Glu Glu Gln Lys Ser Met Gln Asn Lys
85 90

ggtattttat gtcccttaat attacctcat attaaggtgt gtagagtgtt tatttttact 470

gatgggtcaa cttttatatg cacgcatcac tctagaagct cccctctact gtaaataccc 530

gtaacttatt gatcacactt gacatcttct aagtatcata ccagagggtc aagttgtcac 590

ttctgtattg agaaggagtt atattgatct cagctgtttt aacactggtt acactccttg 650

tgtttgcggt tataaacgta gctggtttga tatctgtgta gacagatgac attactgagt 710

gcagagtttt tagagcctct tattgtgatg tagtgtgtgt gaaatggcga gaagcctcga 770

atttacggca cacgtatcaa ttgtaaacac gtatgtccat ggcaaagtct ggattttaag 830

acaccccacc aattggcagg ttgcccaaca ggtttcttcc tccggccgga atttaatgtc 890

tcgtaccagc tatattgttt tatgtactaa tttaggaact ttttgccaaa taaaaaaatg 950

cttgcacctt agctcacttt tttaatgagc atcccagtgc attttgggca tcttgaggaa 1010

ggctttgaca acacttgact aacagacgaa acctaagctc ccacattgtt taaacaacta 1070

gaacacaaga ggtttttgac tcacaacagc atcattccat aaaacaacat tttaaaatca 1130

tgacatgaaa ataggaaatg gaaaaaagga ctatgcatat ttcttgaccg aaactataaa 1190

gatctcttgt agaattaaaa tggattattt taatttggta cgctttccac aaaatgtctt 1250

tttttttttt tgacacaagg gggtccaata tttcaataga gcagcttcac aagccctcac 1310

gagaaatgta aaccaactga caccttcacc tgtacagact gtgcagtaat ctatagtata 1370

tcatcatata gcctaccttg tgataagctt aaatagatgc cttgttaagt tatacacaaa 1430

gttgaatttt gaatattgtg tgcaaataca gaagatgtta ttgaatgttt ttttttcctc 1490

tcgaaataaa attgaccagt cttgtaattc t 1521

<210> 8
<211> 92
<212> PRT
<213> Seriola quinqueradiata

<400> 8
Met Thr Ala Tyr Asp Glu Asn Glu Ser Lys Leu Met Arg Lys Val Lys
1 5 10 15

Glu Asn Pro Phe Val Pro Val Gly Ile Ala Gly Phe Phe Ala Ile Val
20 25 30

Gly Tyr Arg Leu Met Lys Met Lys Asn Arg Gly Asp Thr Lys Met Ser
35 40 45

Val His Leu Ile His Met Arg Val Ala Ala Gln Gly Phe Val Val Gly
50 55 60

Ala Met Thr Val Gly Val Leu Tyr Ser Met Tyr Arg Asp Phe Ile Val
65 70 75 80

Lys Pro Arg Glu Glu Gln Lys Ser Met Gln Asn Lys

85 90

<210> 9
<211> 857
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (236)..(427)

<400> 9
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tcggacgagg gcctcgcaga agggcgggct ttgggaggtc cgtttgtctt tggggcttat 60

ttctatccag agcagtgcct gcgtggagct tccacgttgc gactcagccg accttcttcc 120

ttactcctgc acgacctggt gtgactgtga gcagccgtct ctcaactttt ccttctgagg 180

atctagcagc agaaagcagc tctacttccc tgcaaaggag ctgggcaccg tcgcc atg  238
                                                             Met
                                                              1

aag ttc atg ctg aac ctc tat gtg ctg ggc atc atg ttg acc ctg ctt   286
Lys Phe Met Leu Asn Leu Tyr Val Leu Gly Ile Met Leu Thr Leu Leu
            5                   10                  15

tcc atc ttt gtt aga gtg atg gag tct ctg gga ggc tta ctg gag agc   334
Ser Ile Phe Val Arg Val Met Glu Ser Leu Gly Gly Leu Leu Glu Ser
        20                  25                  30

cca ctg ccc ggg agc tcc tgg atc acg agg ggt cag cta gcc aac aca   382
Pro Leu Pro Gly Ser Ser Trp Ile Thr Arg Gly Gln Leu Ala Asn Thr
    35                  40                  45

cag cct cct aag ggc ctg cca gac cat cca tcc cga gga gtg cag       427
Gln Pro Pro Lys Gly Leu Pro Asp His Pro Ser Arg Gly Val Gln
50                  55                  60

tgaacctccc tccctgcagg catcacagct tcagcatgtc caaccacacg ttccatttct 487

cgggaggcag catcaagtgt ctccaaagga ctcttactag gcctggaagg gctgttccct 547

taccctggaa aagagcctat ttcccctaga gctgtgagtg ggctgtctgt ggctctggga 607

tggaggtgta ccagttccag ctgtagggag aatggatttt ggtttcgttt gtttcagacc 667
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tctgtcctaa aggactcttt tggacctaag tatcttctgt tggtttacca ttgagtctct 727

tccctgagag ttgtttggat ggcatcaaag gggttgtggt ttgactgtga agacagaggg 787

tggactatcc agtgtccagg tcaagttgta catttaagtt ctttctccag tgtaatgcac 847

atgtgtttgt 857

<210> 10
<211> 64
<212> PRT
<213> Mus musculus

<400> 10
Met Lys Phe Met Leu Asn Leu Tyr Val Leu Gly Ile Met Leu Thr Leu
1 5 10 15

Leu Ser Ile Phe Val Arg Val Met Glu Ser Leu Gly Gly Leu Leu Glu
20 25 30

Ser Pro Leu Pro Gly Ser Ser Trp Ile Thr Arg Gly Gln Leu Ala Asn
35 40 45

Thr Gln Pro Pro Lys Gly Leu Pro Asp His Pro Ser Arg Gly Val Gln
50 55 60

<210> 11
<211> 44
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: linker
oligonucleotide

<400> 11
ttttaccagc ttattcaatt cggtcctctc gcacaggatg catg 44

<210> 12
<211> 37
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: linker
oligonucleotide

<400> 12
catcctgtgc gagaggaccg aattgaataa gctggta 37

<210> 13
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: linker
oligonucleotide

<400> 13
tttttgtaga cattctagta tctcgtcaag tcggaaggat gcatg 45

<210> 14
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: linker
oligonucleotide

<400> 14
catccttccg acttgacgag atactagaat gtctacaa 38

<210> 15
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide PCR primer

<400> 15
ccagcttatt caattcggtc c 21

<210> 16
<211> 21

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide PCR primer

<400> 16
gtagacattc tagtatctcg t 21

<210> 17
<211> 32
<212> DNA
<213> Mus musculus

<400> 17
ccgatctaga ggaagggacc ccgcgtctcg ga 32

<210> 18
<211> 34
<212> DNA
<213> Mus musculus

<400> 18
ggcgctcgag tctaaaccca catgttattt attg 34

<210> 19
<211> 21
<212> DNA
<213> Mus musculus

<400> 19
ccttactcct gcacgacctg g 21

<210> 20
<211> 32
<212> DNA
<213> Mus musculus

<400> 20
ggcgctcgag cacatgtgca ttacactgga ga 32